THE BIOCHEMICAL BASIS OF LIVE CELL THERAPY

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Foreword by

Rodrigo Rodriguez, M.D.
Wolfram W. Kuhnau, M.D.

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DEDICATION

The history of medicine and science eloquently teaches that real progress for humanity is made by men and women of foresight and courage who choose to go against the grain, to march to the beat of a different drummer. Human progress is based on their endeavors.

In the area which would finally be denominated "live-cell" or "cellular" therapy, such innovative, independent thinkers have included Alexis Carrel, John Hunter, Charles E. Brown-Sequard, Serge Voronoff and Paul Niehans. And they also include one of the collaborators of the late Dr. Niehans who, more than any other, brought the Niehans approach and cellular therapy in general to the Americas.

This gifted endocrinologist, originally trained in dermatology, has dedicated the better part of his entire adult life to furthering the knowledge and practice of cellular therapy, frequently fighting alone and against great odds for the vindication of this vital medical specialty. For four decades and through many thousands of successful treatments he has painstakingly provided the empirical evidence that live-cell therapy "works."

As a major addition to the international American Biologics and American Biologics-Mexico S.A. Medical Center teams, he has brought a new dimension to individualized, integrated therapies and to the furtherance of the quality he most holds dear: the human spirit.

So, in respectful memory of the lives of Niehans, Carrel and the other live-cell pioneers and in keeping with our desire to acknowledge as well the achievements of the living, we proudly dedicate this book to:

WOLFRAM W. KUHNAU, M.D.

SPECIAL DEDICATION

To my Wife
Carole E. Bradford

without whose continuing support, encouragement and contribution this book would not have been possible

THE BIOCHEMICAL BASIS OF LIVE CELL THERAPY

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FOREWORD

As physicians, we are delighted that for the first time the essential biochemical explanation of the mechanisms of live-cell therapy is finally being presented to the scientific world.

But as physicians we have never required biochemical proof, for we have empirically seen the results of this form of treatment in thousands of patients.

For more than 33 years, Dr. Kuhnau has applied live-cell therapy to somewhere between 15,000 and 20,000 patients in Europe, Africa, Asia and the Americas, his total experience and caseload being among the most impressive in the annals of "fresh" live-cell therapy.

Dr. Rodriguez, as medical director of the American Biologics-Mexico S.A. Research Medical Center and of the Bradford Research Institute (B.R.I.) of Mexico, has seen striking evidence of the effects of live-cell or cellular treatments in his patients as well.

Under his guidance, new vistas for cellular therapy have developed, in particular the addition of pre-treatment and post-treatment programs and the utilization of state of the art cell preparation and preservation including cryoprotectant techniques as well as an expansion of the parameters for testing the sterility, viability and antigenicity of the cells. And the B.R.I. has added the importance of oxidative diagnostics and anti-oxidant therapy as a new dimension in integrated live-cell treatments.

Dr. Kuhnau, who brought the basic Niehans method to the Americas and became the director of endocrinology and collaborator in live-cell studies for the B.R.I. and American Biologics-Mexico, S.A., has seen the role of this form of treatment expanded through a concept developed by the B.R.I.:

Individualized, integrated metabolic programs (I.I.M.P.) in the management of degenerative diseases and metabolic challenges.

This total "I.I.M.P." approach involves a holistic, eclectic mode: the utilization of vitamins, minerals, enzymes, amino acids, oxidants, anti-oxidants and other natural substances together with immunological and endocrinological manipulation in a total-body approach to health, including the conservative integration of standard allopathic diagnostics and practices. The addition of live-cell therapy to this basic concept has enhanced the prospects and capabilities of both.

Within a political setting conducive to innovative medicine and free from the suffocating restraints of a single school of medical thought, we have been free, in Mexico, to pioneer this total approach for the benefit of our patients, who have come to us from all over the world.

We are also fully aware that, however dramatic the live-cell therapy component is in the amelioration and rectification of an ever-widening variety of pathologies, conditions and challenges, it most assuredly is central to another concept strongly emphasized by the Bradford Research Institute:

Promotive health with quality life extension.

The contribution of live-cell therapy to both rejuvenation and regeneration can no longer be seriously doubted. Its prophylactic and rejuvenative use in advance of actual therapeutic need points to genuine utility in retarding the ageing process and in the overall enhancement of the quality, as well as the quantity, of human life.

We have the opportunity to observe the considerable number of positive metabolic effects, some entirely unexpected, obtainable through a broad-based, eclectic program which has been greatly enhanced by the adjunctive administration of live cells.

We are optimistic that time and experience are on the side of live-cell therapy and that the general recognition of its authentic place in the medical universe will not be long in coming.

RODRIGO RODRIGUEZ, M.D.
WOLFRAM W. KUHNAU, M.D.

GLOSSARY

Adipocyte — A specialized cell capable of storing fat.

Alpha-fetoprotein (AFP) — A specific protein prevalent during fetal growth and diminishing with fetal age.

Alpha$_1$-antitrypsin inhibitor — A protein inhibitor of the protein-digesting enzyme, trypsin.

Alzheimer's disease — A disease of the elderly resulting in loss of memory and identity.

Antioxidants — Substances capable of neutralizing the deliterious action of oxygen.

Arachidonate cyclooxygenase — An enzyme which closes and forms a five-membered ring in prostaglandins.

Arachidonic acid — A long-chain fatty acid bearing four unsaturations (carbon-carbon double bonds).

Catalase — An enzyme which destroys hydrogen peroxide.

Cell transformation — A cellular process involving the genes whereby the cell reverts back to a more primitive non-differentiated state, typified by cancer cells.

Chelation (chemistry) — The binding of metals by organic groups (ligands).

Chelation (clinical) — The binding and removal from the body of excess calcium through the use of the chelating agent, EDTA.

Chlorella — A specific single-cell algae.

Cholesterol — A specific steroid usually found within the plasma membrane for the purpose of decreasing membrane fluidity.

Cortisone — A naturally-occurring steroid having antiinflammatory properties.

Cryopreservation — A process for preserving or storing cells while kept under extreme cold, usually liquid nitrogen.

Cyclic AMP (cAMP) — A modified form of the nucleotide, adenosine monophosphate (AMP), in which the phosphate group has cyclized (condensed) with one of the hydroxy groups of ribose. A second messenger for many hormones.

Cytosolic receptor — A receptor found in the cytosol of the cell (internal).

Cytotoxic cells — Certain white blood cells capable of killing other cells.

Dexamethasone — A steroidal antiinflammatory drug.

Diacylglycerol — A diester formed from glycerol (glycerine) and two fatty acids.

Differentiation — A cellular process characterized by development and specialization.

DNA (deoxyribonucleic acid) — One of the two nucleic acids formed as a chain of four basic units forming the genetic material of a cell.

Down regulation — The process by which cell surface membrane receptors are internalized and either recycled to the surface or destroyed by lysosomes.

EDTA (ethylenediaminetetraacetic acid) — An organic chelation agent capable of binding and removing excess calcium from the body.

EGF (epidermal growth factor) — A polypeptide having an approximate molecular weight of 6000 which stimulates activity in a wide variety of cells.

EPA (eicosapentaenoic acid) — A long-chain (20-carbon) fatty acid having five unsaturations (carbon-carbon double bonds).

Epinephrine (adrenalin) — A simple organic compound acting as a neurotransmitter.

Erythrocyte — Red blood cell (RBC).

Fibroblast — A specific cell type quite often found in the skin.

First messenger — A hormore or biological signaling agent which conveys a message from one cell to another in a distant part of the body.

Free radical — A chamical grouping or piece of a molecule bearing an unpaired electron (highly unstable).

Freeze-dried cells — Cells which have been frozen and then subjected to a vacuum, resulting in the evaporation (sublimation) of ice.

Galactose — A specific sugar (hexose) found in polysaccharides immediately adjacent to terminal sialic acid.

Genetic Code — The set of all nucleotide triplets, each of which represents a single amino acid.

Glomeruli — The basic functional unit of the kidney which separates useful materials from waste products of body metabolism.

Glutathione peroxidase — An enzyme which destroys hydrogen peroxide in conjunction with the tripeptide, glutathione.

Glycoprotein — A protein covalently (permanently) attached to polysaccharide chains.

Glycosylation — A biochemical synthetic process following protein synthesis during which polysaccharides are permanently attached to protein.

Golgi body — A subcellular membraneous organelle continually releasing small vesicles resembling lysosomes.

Haber-Weiss Reaction — A chemical reaction in which superoxide combines with hydrogen peroxide resulting in the formation of hydroxyl radical.

Histamine — An organic substance capable of altering the permeability of membranes to water.

Histones — Basic (alkaline) proteins consisting largely of lysine and arginine which mask DNA and control genetic activity.

HLB Blood Test — An easy to perform blood test made from a single drop of blood which is allowed to dry on a microscope slide and examined.

Hormone — A substance, usually of low molecular weight, sending a message between cells in distant parts of the body.

Hydrogen peroxide — A molecule of water containing one additional atom of oxygen (H_2O_2).

Hydrophilic — A material or substance compatible with water and binding to it.

Hydrophobic — A material or substance incompatible with water, repelling it.

Hydroxyl radical — A free radical consisting of the grouping, OH (symbolized by $OH^{\bullet}$).

Hypothalamus — A portion of the brain used by live cell therapists.

IgE — A specific immunoglobulin participating in allergic reactions.

IgG — Immunoglobulin G, one of a class of antibodies forming the humoral immune system.

Inositol — One of a class of polyhydroxy six-carbon organic rings having significance in phosphorylation processes.

Interferon — A high molecular weight natural substance related to combatting viral infection.

Interleukin 2 — A naturally occurring peptide having immunological significance.

Lectin — A protein capable of binding to specific polysaccharides on glycoproteins.

Limbic system — Closely related portions of the brain which result in the expression of certain emotions such as anger, fear, etc., basic to survival.

Liposome — A small subcellular synthetically prepared spherical membrane containing any desired substance in solution, resembling a lysosome.

Log phase (cell culture) — That early phase of cells growing under culture conditions in which the number of cells increases logarithmically.

Lymphocyte — A population of white blood cells having several subpopulations highly significant in the immune defense system.

Lysosome — Small subcellular sacs consisting of a bilayer lipid membrane enclosing digestive enzymes.

Mannose — A 6-carbon sugar (hexose) having structural similarity to glucose and highly significant in relation to those receptors bearing polysaccharides.

Mast cell — A specific white cell usually found in skin capable of releasing histamine with proper stimulation.

Membrane fluidity — The degree of random motion among the lipid and other components of the plasma membrane.

Membrane, bilayer lipid — A molecular film consisting of two opposing layers of lipid in which proteins are imbedded.

Microtubule — A straight chain of specific subunits (tubulin) which may form and disintegrate for the purpose of moving subcellular organelles within the cell.

Molybdinum — A little-known mineral (metal) which has been shown to have significance in nutrition.

Myristic acid — A 14-carbon saturated long-chain fatty acid found in nutmeg and pollen.

Natural killer cells (NK cells) — A certain population of white blood cells capable of killing other cells.

Neuraminidase (sialidase) — An enzyme capable of removing sialic acid from the terminal ends of polysaccharides.

Nucleotide — One of the fundamental units comprising the nucleic acids, RNA and DNA.

Octylglucoside — A non-toxic detergent employed in the extraction of specific subcellular components.

Osmosis — The passage of water through a semipermeable membrane from a solution of lower concentration to one of higher concentration.

Periodic acid — An oxidizing agent in which iodine is highly saturated with oxygen, capable of being released as atomic oxygen.

Peroxidation — The binding of oxygen to a ruptured carbon-carbon double bond, usually found in an unsaturated fatty acid.

Phagocytosis — The process by which small particles are ingested by certain white blood cells.

Phosphoprotein phosphatase — An enzyme which dephosphorylates protein.

Phosphorylation — The process of binding phosphate groups to amino acids bearing a hydroxy group.

Phytohemagglutinin (PHA) — A protein isolated from plant sources capable of stimulating lymphocytes.

Plasma membrane — The outer bilayer lipid membrane of a cell.

Polymer — An organic substance formed by linking together into a chain a few types of fundamental units.

Polypeptide — A short-length protein of low molecular weight having little or no secondary structure.

Polysaccharide — Molecules consisting of specific linkages of sugars.

Polyvinylprrolidone (PVP) — A polymeric substance capable of binding large amounts of water.

Post-therapy, fetal cell injection — Therapy given a recipient of live fetal cells following the injection for the purpose of obtianing a greater and more prolonged response.

Pre-therapy, fetal cell injection — Therapy given a recipient of live fetal cells prior to the injection for the purpose of obtaining a greater response.

Prostaglandin — A hairpin-shaped molecule formed from arachidonic acid acting as a second messenger in hormonal expression.

Protein kinase — An enzyme which phosphorylates protein on specific amino acids.

Quercetin — A sugar-carrying derivative of rutin.

Receptor — A protein or glycoprotein capable of binding and responding to a specific substance.

Retinoic acid — A derivative of vitamin A having an unsaturated hydrocarbon chain terminating in a carboxylic acid.

Reverse transcriptase — An enzyme arising from viral activity capable of replicating DNA from RNA.

Ribosome — A subcellular body (organelle) composed of several subunits acting as a support and catalyst in protein synthesis.

RNA (ribonucleic acid) — One of the two nucleic acids formed as a chain from four basic units.

Sialic acid — A specific sugar derivative usually found on the terminal ends of polysaccharide chains.

Sialidase (neuraminidase) — An enzyme capable of removing sialic acid from the terminal ends of polysaccharides.

Sodium butyrate — The sodium salt of the 4-carbon organic acid, butyric (butter) acid.

Sublimation — The conversion of a solid to a gas without passing through the intermediate liquid phase, usually conducted under vacuum.

Superoxide — Molecular oxygen which has acquired an additional electron as a result of breaking the oxygen-oxygen bond (O_2^-.).

Superoxide dismutase — An enzyme which destroys superoxide.

SV-40 — A virus (simian virus) used in biological research.

Transferrin — A specific serum protein capable of transporting iron.

Trypsin — An enzyme capable of breaking (digesting) protein (proteolytic enzyme).

Tuftsin — A naturally occurring tetrapeptide cleaved from immunoglobulin G (IgG) having immunological significance.

CHAPTER I.

INTRODUCTION

For six decades a dedicated growing group of international physicians has been achieving impressive results in an ever-widening array of pathological conditions and metabolic challenges through the intramuscular, and later on subcutaneous, injections of suspensions of animal fetal cells.

This form of therapy, essentially organized and pioneered by the late Paul Niehans, M.D., of Switzerland (though he built on the considerable conceptual and clinical work of, among others, Alexis Carrel) came to be known as "live-cell" or "cellular" therapy.

While live-cell treatments came to be relatively well known and medically accepted in Europe — research involving a Nobel laureate as well as a number of other medical and scientific notables — the biochemistry of just how cellular suspensions were capable of treating everything from eczema to advanced degenerative diseases of all kinds, including cancer, has up to now largely been speculative. Because the notable achievements of live-cell therapy, now numbering in the hundreds of thousands of cases, have been empirical observations without a bolstering biochemical explanation, American-oriented allopathic medicine has largely ignored or opposed this therapeutic approach.

The vast majority of medical and biochemical literature on this subject is in German and other European languages, with but a trickle making its way into English. Because of the generally hostile attitude of the American allopathic medical establishment, its considerable influence in the Western world, and the paucity of clinical research available in the English language, the English-speaking world — aside from an elite of statesmen and notables in

the circles of art and culture — has generally been ignorant about live-cell therapy.

Now, however, a search of the world literature on all aspects of live-cell therapy, supported by the actual clinical evidence of its multiple effects as well as recent research in cellular biochemistry, has provided the authors with the opportunity of presenting, for the first time, the actual biochemical rationale for and explanation of this modality.

It is appropriate to note that, whatever name it is given, this general approach is coming into its own, even when viewed by American orthodoxy. As early as 1970, Trainin and Small[1] showed the extracts of calf thymus conferred immunocompetence on lymphoid cells in mice, an outcome that any experienced live-cell therapist would have expected. In 1981, Osband et al.[2] detailed how 10 of 17 children treated for the immunosuppressive condition called histiocytosis-X underwent complete remission after being treated with daily intramuscular injections of thymus extract from five-day-old calves.

Until the advent of radioactive tagging, live-cell therapists could only rely on empirical evidence to prove they were getting results with cellular therapy in accordance with the postulates of Paracelsus and (oddly enough) Hahnemann — "like cures like," that spleen cells from animals affect the human spleen, that brain extracts from calf embryos affect cerebral tissue in man. Modern research which strongly supports the empirical evidence has come from such groundbreaking research as that of Schmid and Lettre of the University of Heidelberg and Professor A. Kment, University of Vienna. Dr. Schmid showed in animal tests that immediately following injection cellular groups and tissues from donor animals are transported in the host's blood to counterpart organs and tissues.[3] Lettre used material tagged with radioactive isotopes — and a Geiger counter — to prove that cellular material did in fact

reach the target organs and tissues. Weiss at the Rockefeller Institute[4] conducted experiments which demonstrated the "self-organization" capacity of cells so that information contained within specific kinds of cells reacts in such a way that the mass of cells become identifiable as tissues — that is, heart cells link to each other to form rhythmically contracting tissue.

Even before the decade of the 1980s, live-cell therapists had demonstrated the utility of their therapeutic in the folloiwng diseases, conditions and challenges:

- Neuromuscular disorders, including epilepsy, multiple sclerosis, amytrophic lateral sclerosis (ALS), Parkinson's, post-stroke paralysis and muscular dystrophy.
- Hormone-dependent dysfunctions including a full range of sexual disorders ranging from impotence and early menopause as well as obesity, insufficiency and hypothyroidism.
- Chronic dermatological disorders, especially psoriasis and eczema.
- Chronic arthritis of all kinds.
- Chronic pancreatitis.
- Arteriosclerosis.
- Liver cirrhosis.
- Allergies of all kinds.
- Genetic and hereditary disorders, including mental retardation, Down's syndrome, bone and cartilage abnormalities, congenital hip malformations, congenital dysplasias, spinal problems, cleft lip and palate.
- Chronic lung disease.
- Chronic kidney disease.
- Auto-immune disease.
- Narcolepsy.
- Rejuvenation.

In the 1980s, and with the advent of the bringing of the Niehans system to Mexico (primarily through one of his disciples, W. W. Kuhnau), cellular therapy was fitted into a broader concept by the Bradford Research Institute in conjunction with American Biologics-Mexico, S.A. Research Medical Center. This broader concept is "individualized, integrated metabolic programs" (I.I.M.P.) — a holistically oriented and eclectic multidisciplinary approach to chronic, systemic degenerative diseases.

The new dimension afforded vast new vistas for the Niehans method, greatly expanding its capabilities in the areas of rejuvenation and regeneration and allowing live-cell therapy to play a role in degenerative diseases, in cancer therapy and in such lethal new challenges as acquired immune deficiency syndrome (AIDS).

But in whatever program live-cell therapy is used, the treatments are still basically aimed at a combination of endocrinological stabilization ("hormonal harmonizing," as Dr. Kuhnau calls it), rejuvenation and regeneration. All such treatments consist of the intramuscular injection of cellular suspensions from embryonic endocrine glands and other tissues, the injections given in rapid sequence in one sitting. The combination of which kinds of cells to give constitutes not only the "science" but the "art" of live-cell therapy: the use of thymus, brain, and adrenal live cells in muscular dystrophy, or of bone and alveolar cells for arteriosclerosis, for example. While as many as 40 kinds of glandular or tissue cellular suspensions are normally available for live-cell therapy, it is rare to give more than six in one day for a single course of treatment.

The combining of live-cell injections within a total program of vitamins, minerals, enzymes, amino acids, anti-oxidative substances and dietary manipulation, as advanced by the B.R.I.-AB-Mexico effort, began turning in startling results in the 1980s, which continue to the present time. The addition of a thorough pre-

treatment program to potentiate live cells is a primary element in this new dimension, as pioneered by the B.R.I.

The cellular suspensions provide some results which may be felt almost immediately and others, more typically, become apparent from three to five months later. And as results appear, patients almost always experience a wide variety of positive responses. Not only do specific pathologies reflect benefits, but such subjective signs as sudden improvement in energy levels, vision, appetite normalization, immunological panels and sexual performance are frequently reported.

For the first time it is now understood why and how these changes are being effected by live-cell therapy.

In the following account, the authors will discuss the historical development of live-cell therapy and the central biochemical aspects of it:

- How injected cells stimulate the immune system.
- The various biochemical mechanisms involved in the process, with particular attention to cell recognition receptors.
- The vital role of alpha-fetoprotein, a specific protein produced by fetal cells.
- Cellular extraction and preservation.
- Cryopreservation and cell culture.
- How some cell preparation techniques lead to damaging immunological and oxidative effects.

History

Both live-cell therapists — in the main allopathically minded MDs — and homeopaths, ironically enough, can trace the ideological origins of their particular approaches to Paracelsus of the 16th Century. This philosopher/mystic/scientist is said to have said:

"Similia similibus curantur" — roughly translated, "like cures like."

Both camps would now say that their divergent departures from Paracelsus are irreconcilable, the homeopaths still favoring minute dilutions of substances which produce disease-like symptoms in healthy people being used to treat the unhealthy, the live-cell therapists favoring macrocellular isolations of glands and tissues from animal embryos. But both clash with the "solidist" post-Galen doctrine which has come to dominate Western medical thought. For many years, both homeopaths and live-cell therapists could empirically report on what they were doing yet not know why what they were doing "worked."

At least in the area of live-cell therapy, we now know why -- as presented in the present text -- this particular approach "works."

While the late Swiss physician Paul Niehans, M.D., is usually accorded the title "father of live-cell or cellular therapy," indeed he was preceded by other investigators and clinicians in his own century and by certain therapeutic approaches from ancient times. It was Dr. Niehans' popularization of live-cell or cellular therapy which, more than anything, catapulted his name into global prominence as the primary exponent of this particular innovation. Yet he neither created nor ideologically conceived the approach which was to make him famous.

Indeed, the oldest medical document extant, the Eber papyrus (c. 1550 B.C.) refers to medicinal preparations made from animal organs. We know that as early as 1499 B.C. a Hindu physician named Susrata recommended the eating of tiger gonads as a way to cure impotence, and that the ancient Chinese prescribed human placentas as a tonic. There are many references to the therapeutic use of organs in the Materia Medica of Aristotle and Pliny the

Elder. And Homer wrote how Achilles consumed the bone marrow of lions in order to stimulate his own strength and bravery.

Indeed, it is generally accepted that, at least in Mesoamerica and New Guinea, the indigenous tribes which practiced cannibalism reflected a relatively high degree of civilization and that the living organs consumed by the victors came from the vanquished who were considered to be the best-quality prisoners in terms of bravery and strength. Whether this was pure ritualism or reflected some transient biological reality we may never know, but we strongly suspect the latter, given important research breakthroughs in the late-20th Century.

In the 18th Century, Scottish physician John Hunter demonstrated the stimulation of sexual regeneration in a castrated capon by grafting rooster testicles onto it, an experiment repeated a century later by A. A. Berthold. In 1889, Charles E. Brown-Sequard of the French Academy of Medicine took injections of dog testicles and reported several positive results. The utilization of sex glands to stimulate rejuvenation was by no means a total novelty by the end of the 19th Century, though the over-emphasis on restored sexual vigor through a primitive form of cellular therapy deflected interest from the far greater role of such therapy in degenerative disease in general, a reality which continues to the present time.

By the end of the century, Dr. Serge Voronoff of Russia had become particularly noted for the implantation of strips of chimpanzee testicles in ageing men. But two far more serious approaches to what would become modern cellular biology were percolating: Virchow adduced the concept of cellular pathology, identifying the unitarian completeness and singularity of the individual cell; and the French-American surgeon Alexis Carrel established the foundation of cell culture.

If any single man in this century can be more adequately thought of as the actual "father" of live-cell therapy, it should be

Dr. Alexis Carrel (1873-1944), despite his greater fame as a Nobel Prize winner (development of a new technique for sewing up blood vessels end to end). Following the pioneering work of Ross Harrison at Johns-Hopkins University, Dr. Carrel also successfully refined techniques of tissue culture research. Primarily associated with the Rockefeller Institute for Medical Research, Dr. Carrel became intimately involved with the early work on organ transplants, ultimately transplanting a kidney from one cat to another.

His greatest breakthrough, from the cellular therapy aspect, was his project in January 1912 in which he succeeded in transplanting heart tissue from a chick in an in vitro culture — and maintained the culture in a living state for an astonishing 32 years. It was his work in keeping the fragments of a chicken heart alive for many years after the fowl's death that had a particular impact on Paul Niehans, M.D., who later collaborated with the Franco-American surgeon and developed what would later become known as live-cell or cellular therapy.

Wolfram Kuhnau, M.D., the Niehans disciple who later became the "bridge" between European live-cell therapy and the new era of this approach as pioneered in Mexico, has recalled in his own monograph[5] that "the technique of injection-implantation was first reported in 1912 by Kuttner but forgotten for a long while. In 1927, Kurtzahn and Hubener published a major work on thyroid implantation by injection in the treatment of myzedematous children. In 1929 Kuttner again published, this time on the injection-transplantation of endocrine glands. What Niehans originally did, independent of the earlier research, was in effect to rediscover Kuttner's procedures, greatly amplify them and develop a comprehensive treatment technique.

Dr. Kuhnau had noted the Kuttner paper in 1929 but was not interested in it at the time, but later, through a series of

events, found himself working along the same lines as Dr. Niehans, though independently. Dr. Kuhnau, originally trained as a dermatologist, became intrigued with the idea of implanting whole pituitary glands from calves under the skin of patients with chronic dermatological problems.

He recalls: "In the forties, we knew enough about the immune system to understand that only glands from unborn animals should be targeted for such experimental therapy, for they would be tissues developed before the maternal immune response was in place. In continuing efforts to eradicate persistent skin diseases with hormone therapy, it was in the late forties that I attempted to implant these whole pituitary glands." This method had been developed by a Dr. Fellinger of Vienna. Yet there was no observable effect, so Dr. Kuhnau turned to grinding up the glands and pulverizing them into a paste, then placing them in isotonic salt solution to prevent autolysis, then injecting the whole substance intramuscularly. Only then did he see effects.

"And what effects!" he wrote. "I was able to see the rapid diminution and eradication of a whole range of skin disorders and allergies. I published these results in 1952. I simply did not know at that time, through trial and error, I had used exactly the same method already being used by Dr. Niehans."

On the basis of the Kuhnau paper, Dr. Niehans invited the Breslau native to Switzerland. Out of their collaboration came Dr. Kuhnau's lifelong dedication to cellular therapy, which, as of this writing involves more than 33 years of experience with between 15,000 and 20,000 cases.

Niehans developed and scientifically promoted the theory and practice of utilizing "fresh" live cells from animal embryos. But he had not proceeded too far before becoming popularly better known as a therapist who could bring about rejuvenation and sexual

restimulation than a physician who could manage a number of conditions with live cells.

Dr. Niehans has written[6] of the beginnings of live-cell therapy:

"Professor Quervain, of Berne, sent to me for a parathyroid graft a patient with severe post-operative tetany, which up to then had given no response to any therapeutic measures. As the patient arrived in a moribund condition, I could not even consider a surgical implantation. So I cut the parathyroid glands of an ox into tiny pieces, made a suspension with physiological saline solution, and injected it into the pectoral muscles of the patient.

"I thought the effect would be short-lived, just like the effect of an injection of hormones, and that I should have to repeat the injection. But to my great surprise, the injection of fresh cells not only failed to provoke a reaction, but the effect lasted, and longer than any synthetic hormone, any implant or any surgical graft. Twenty-six years have passed and the patient is still free of cramps."

Dr. Niehans has described how the implantation of organs gradually gave way to the implantation of organs by injection. By 1937 he was using tissue cultures from both sick animals and humans, his work interrupted by World War II but continued with Dr. Wirth at the University of Geneva Institute of Pathology. Along the way, even though Dr. Niehans' approach was frequently attacked by more orthodox allopathic physicians, he interested such investigators as Nobel Prize winner Kment from Vienna, Landsberg of Heidelberg and Rosenkranz from the U.S.A. in his methods, and even Josef Stalin of the U.S.S.R.

Dr. Kuhnau records that in 1948 Niehans collaborated with a Professor Bauer of the Clarens Clinic in Switzerland in studying the therapeutic effects of preserved cells, and in 1949 conducted

research with Professor Pischinger into the cancer-resisting properties of fresh cells. He later developed the freeze-drying process of fresh cells, which was to produce one of two important deviations in live-cell history.

Scientific researchers from several countries began working along the lines suggested by Dr. Niehans, and by the mid-1960s papers from investigators in many lands all dealing with aspects of cellular therapy were published. By now, Niehans and his approach were famous.

As Kuhnau has noted: "Political and state leaders who received the benefits of cellular therapy have included . . . Konrad Adenauer, Charles DeGaulle, Dwight D. Eisenhower, Sir Winston Churchill, the Duke and Duchess of Windsor, Haile Selassi, the monarchs of Morocco and Saudi Arabia, Bernard Baruch, and Joseph Kennedy." Other notable recipients of the benefits of cellular therapy also included such actors and actresses as Charlie Chaplin, Robert Cummings, Gloria Swanson, and Paulette Goddard; artist Pablo Picasso; playwright Noel Coward; novelist Somerset Maugham and perhaps the most famous Niehans patient of all -- Pope Pius XII, who, while dying, summoned Niehans to his bedside for injections and went on to live for four more years.

Journalists and lay observers, however, tended to concentrate on the increase of sexual potency and the longevity aspects of live cell therapy so that by the late 1960s, at least in the Americas, the Niehans approach was thought to deal with those matters almost exclusively. The over-emphasis on longevity and restoration of sexual vigor, however genuine, represented the first major deviation in establishing this therapeutic modality as legitimate.

A second fork in the road, which continued today, was the entry of lyophilization into the live cell preparation process. Again quoting Niehans' disciple Dr. Kuhnau:

"This regal man (Niehans) was a great idealist whose aim was to make live cells available to thousands of doctors. His dream was to create a product by lyophilizing — freeze-drying —cellular suspensions. These freeze-dried cells would be made available to the world through pharmaceutical companies. My objection then, as now, is that freeze-dried cells are definitely not 'live' cells. Visionary that he was, Dr. Niehans' dream was right — only his projected method was wrong. He did confide his fear to me that those who became more involved in the development of easily available live cells might become more stimulated by the profit motive than by honest medical research."

Subsequent research has borne out much of what Dr. Kuhnau said:

Live cells must, for all practical purposes, be preserved in some manner for future use. Freezing and freeze-drying became the two methods for cell preservation, but the specific manner in which cells are frozen and thawed determines whether they will be viable following storage in the frozen state. It is now understood that typical freeze-drying not only ruptures cells, but inactivates cell recognition receptors and liberates much more antigenic material than from whole cells, and that freezing without cryo-protectants and timed-freeze and thaw techniques alterscells, preventing them from synthesizing the important alpha-fetoprotein (AFP) and other cellular materials.

Cell preservation problems have been solved. The B.R.I. is researching advances in the modern techniques of cell culture which promise to replace present-day methods of cell preservation.

Dr. Niehans died in the early 1970s, but several of his major disciples have carried on his work, extending it into more fields than ever before. A key one of these has been Dr. Kuhnau, who brought the Niehans technique to Mexico in 1980.

The German physician became the major live-cell and endocrinological consultant for American Biologics-Mexico S.A. Research Medical Center, which under the direction of Dr. Rodrigo Rodriguez had recently been established to utilize individualized, integrated metabolic, nutritional and eclectic-medicine programs in the management of chronic, systemic degenerative diseases.

Working in collaboration with the Bradford Research Institute of the United States and Mexico, AB-Mexico physicians, under the medical directorship of Rodrigo Rodriguez, M.D., advanced "holistic" programs for a significant number of degenerative conditions in which live-cell therapy plays either a central or adjunctive role. Their research has shown, for example, that the administration of anti-oxidants potentiates live-cell therapy.[7]

The fitting of cellular therapy into a rational, individualized, eclectic medical program has thus secured for this form of treatment its major role in the medicine of the future.

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CHAPTER II.

MEMBRANE RECEPTORS IN CELL THERAPY

PART I

Cell Recognition and Adhesion Through Organ-Specific Surface Receptors

INTRODUCTION

Cell membrane receptors are modulated by phosphorylation processes, membrane fluidity, reactive oxygen toxic species and the classical mediators of biological signals. The multitude of receptors are critical to cellular activity. Many observations made over the years by both clinicians and researchers are cited and given explanation. The elucidation of the biochemical processes facilitates both pre- and post-therapeutic intervention to optimize the systemic environment critical to live cell therapy. This chapter on membrane receptors is divided into six parts. In Part I the topics covered are:

1. The specific binding of a given cell type (from a particular organ) with the organ of the same type (for example, fetal pancreas cells binding to human pancreas), known as "targeting."

2. The non-specific binding of a specific cell type to a different organ (for example, fetal pancreas cells binding to human liver).

3. The importance of the age of the embryo in relation to cell binding specificity.

4. The long-term effect of live cell therapy.

5. Evidence that cells from a specific organ of a donor animal (fetus) will specifically bind to human cells of the same organ.

6. Evidence that a minimal inflammatory response (including digestive processes) following a live cell injection does

not destroy the ability of the cells to exert a beneficial effect on the target organ.

7. Evidence for the marked improvement seen with live cell therapy in neurological diseases such as Mongolism, Alzeimer's disease, multiple sclerosis and amyotrophic lateral sclerosis (ALS). The stimulation of Schwann cell growth in culture through cellular contact confirms the results of live cell therapy seen clinically.

8. Suggestions for improvement in the technique of live cell therapy as presently practiced.

9. The significance of RNA.

10. The implications of cell surface recognition receptors in cancer therapy are discussed.

11. The significance of reactive oxygen toxic species to cell therapy.

THE PLASMA MEMBRANE

Cellular membranes are extremely significant structures found both within and on the surfaces of cells. All cellular membranes are composed of two layers of lipids and, for this reason, are known as "bilayer lipid membranes." Lipids are organic molecules consisting of long chains of carbon atoms which are incompatible with water (hydrophilic). In the membrane the long hydrocarbon chains lie perpendicular to the plane of the membrane with the two free ends of each layer touching in the middle. The hydrophilic or "polar" groups are in contact with water at each side of the membrane (see Fig. 1). The membrane surrounding a cell is known as the plasma membrane.

Imbedded in this membrane are proteins (see Fig. 2) which may project (1) on the side external to the cell, (2) on the internal side of the cell or (3) may extend completely through the membrane

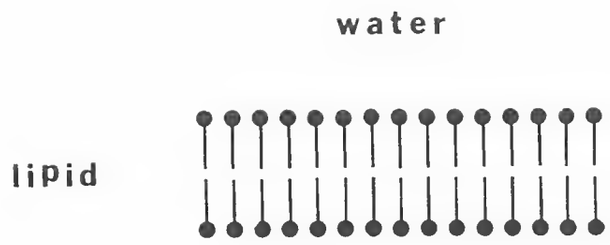


Fig. 1

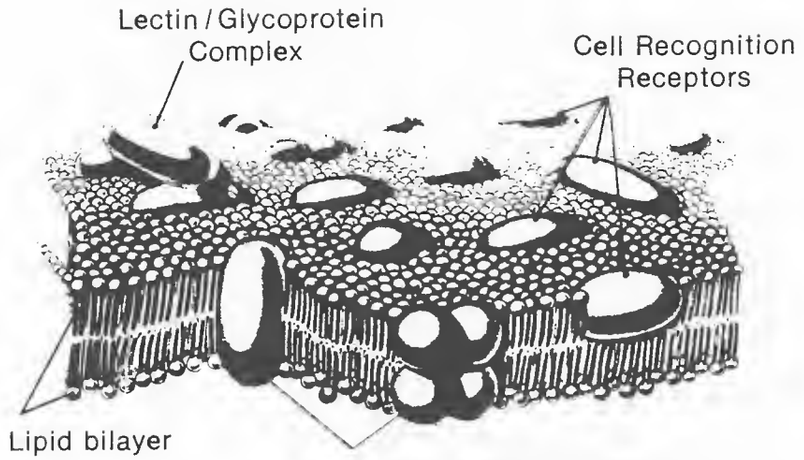


Fig. 2

and project from the surface on both sides of the membrane. It is possible for these proteins to be imbedded in the membrane because they (1) have hydrophobic amino acid residues on their surfaces which contact the hydrophobic hydrocarbon chains comprising the membrane, (2) have hydrophilic amino acid residues on the surface which extend into the water phase and (3) have attached to certain specific amino acid residues long-chain hydrocarbons which are compatible with and bind to the hydrocarbons comprising the membrane. These enzyme-bound hydrocarbon chains are associated with specific hydrocarbons (lipids) present in the membrane which partly determines the activity of the protein. Thus, the particular lipids comprising a membrane partly determine the activity of proteins (enzymes included) found within it.

Some of these membrane-bound proteins are enzymes while others are receptors for hormones or macromolecules for cell recognition and adhesion (to be discussed more fully below).

Some membrane-bound proteins (receptors) are mobile and may move laterally through the membrane by means of the subcellular organelles known as "microtubules." These are long chains of a subunit protein known as "tubulin." The length of microtubules may change rather rapidly, either increasing or decreasing, resulting from the addition of tubulin to the chain or its removal, respectively.

The mobility of membrane-bound proteins is also regulated by what is termed the "fluidity" of the membrane. This characteristic of membranes is related to the freedom of motion of the fatty acids comprising the lipids, resulting from thermal agitation. This in turn is related to the degree of unsaturation in these fatty acids as well as the amount of cholesterol in the membrane. It is believed that one function of cholesterol is to stabilize the bilayer lipid membrane (lower its fluidity). Excessive cholesterol, however, may lower membrane fluidity to the point of impairing

the lateral motion of cell recognition proteins thereby being detrimental to normal cellular activity. The importance of rapid lateral mobility of the receptors is related to the necessity, if cell binding is to occur, for a precise alignment of the receptors present in two opposing plasma membranes.

The fluidity of the membrane is adversely affected by the production of certain Reactive Oxygen Toxic Species (ROTS) by peroxidizing the unsaturated fatty acids and will be more completely discussed below (see Cell Recognition and Adhesion -- Relationship to ROTS).

GLYCOPROTEINS — LECTINS

Some of the proteins present in the plasma membrane are bound to chains of sugar molecules known as polysaccharides. Proteins bound to polysaccharides are known as "glycoproteins."

Specific proteins isolated from the seeds of certain plants are known as "lectins" and have cavities in their structure which are specific for particular sugars. Other specific proteins capable of binding carbohydrate (polysaccharide) may be described as lectins even though not isolated from a plant.

All multicellular animals contain on the surfaces of their cells specific glycoproteins as well as specific lectins which have specificity for the glycoproteins thereby binding the cells together. See Fig. 3.

The lectins isolated from plant seeds (the best known is "concanavalin A" or simply Con A) are "polyvalent," that is, each lectin carries on its surface more than one binding site for carbohydrate. With this type of structure it becomes obvious that the lectins will act as cell agglutinating agents (forming cellular aggregates or clumps). This is true because each lectin molecule may

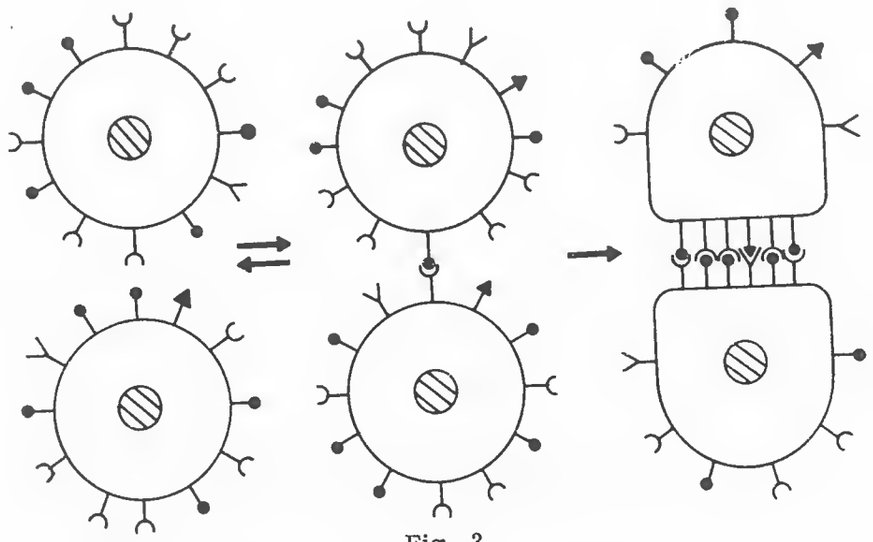


Fig. 3

bind two different cells carrying the appropriate glycoproteins. Thus, cells are "crosslinked" by Con A and become a "polymer of cells" or an "aggregation."

CELL RECOGNITION AND ADHESION

That cells have the ability to recognize other cells of their own type and adhere to them was first demonstrated in 1907 with marine sponges. Several species of sponges, each of a different color, were mechanically dissociated into individual cells and mixed. After a period of time discrete organisms formed, each consisting of cells of a single color (and therefore, species).[1] Other model systems which have been studied to reveal more about cellular recognition and adhesion are sea urchin fertilization, reaggregation of dissociated chick embryo cells and cultured fibroblasts and Schwann (nerve) cells. A steadily increasing body of evidence

indicates that cells contain on their surfaces the means to specifically recognize and bind to other cells.[1] Many excellent reviews have been published describing research in this area.[1-5]

As mentioned above, each cell has present on the plasma membrane two types of receptors, (1) a carbohydrate-carrying protein (glycoprotein) and (2) a protein (lectin) having binding specificity for the carbohydrate portion of the glycoprotein. Cells from a defined anatomical region (a specific organ) prefer to adhere to each other as compared to cells from a different anatomical region. This is obvious from the fact that cells in a given organ remain together at the end of the developmental process.[1] Adhesion of cells is, however, not an "all or none" phenomenon. It has been observed in the chick embryo system that most dissociated embryonic cells will stick to nearly all other cell types from the embryo. With time the cells tend to sort out within these random aggregates to form islands of rather homogeneous cell type.[1]

The first step in cell recognition is thought to be reversible followed by one or more "irreversible" reactions. It is also believed that the irreversible steps may eventually be reversed to account for cell migration and other adjustments during development. This change in adhesive specificity may be accomplished through selective destruction of the adhesive sites.[1,6] See Fig. 4.

Nonspecific macromolecules have been isolated from chick embryo fibroblasts that can rapidly agglutinate several different types of dissociated embryonic cells.[7] Its presence in such large amounts on the cell surface (3% of the total cell protein) indicates a role for this protein in general cell-to-cell adhesion.

These cellular effects, seen with dissociated cells in the laboratory, are in complete agreement with observations made in live cell therapy. It has been noted that there is a greater binding of fetal cells of a given organ to that same organ in the recipient

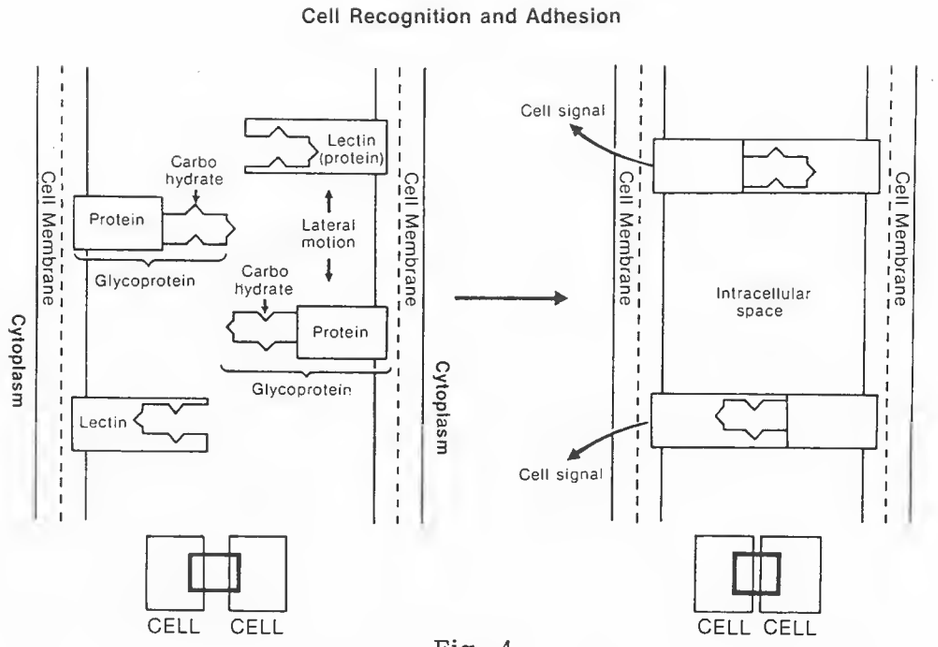


Fig. 4

than to other non-related organs.[8,9] It has also been noted that this selectivity or "targeting" effect is not perfect but that fetal cells of one organ may be found in other non-related organs of the recipient.

In many instances dissociated cells from the same organ but from different species will coaggregate.[10-12] One exception to this is liver cells, indicated by the lack of chick and rat hepatocyte binding.[13,14] This result implies that fetal liver cells from a donor animal may have less specificity for human liver than cells from other fetal organs and may be meaningful in live cell therapy using fetal liver.

Embryonic chick muscle contains two lectins which show changes in activity with development. The first is present in very

low levels in 8-day old chick embryos, rises to a maximum in 16-day embryos and then declines to very low levels in the adult.[15] At its maximum it constitutes about 0.1% of the protein in muscle extracts.[16]

In experiments with mixed, dissociated chick and mouse embryonic cells there was initially a random distribution of all cell types which, with time, sorted out to form the normal histological pattern characteristic of the tissue from which the cells were originally obtained. The aggregated cells of one tissue type proceeded to undergo the tissue-specific cell pattern formation and differentiation.[17]

In similar experiments mixed aggregates containing cells that had been dissociated from two different types of tissue formed a random distribution of all the cells. Further culturing resulted in the sorting out of the cells according to tissue type.[18,19]

These experiments indicate that embryonic cells have specific affinity and exhibit selective adhesion for cells of the same type. In such experiments it is invariably observed that in freshly dissociated mixed cell types (from different organs of the same species) aggregates of only one tissue type occur at a greater rate than the formation of aggregates from two different tissue types.[20,21]

In such experiments trypsin-dissociated cells show a lag period before they attain their maximal rate of adhesion.[22,23] During the recovery period cell adhesion may be prevented with inhibitors of protein synthesis.[23] This implies that certain proteins on the surface of the membrane damaged by trypsin must be replaced before cell adhesion may occur. There is additional evidence that tissue-specific macromolecules are present on cell surfaces that normally prevent the formation of adhesions between different types of tissue.[24]

Relationship to Reactive Oxygen Toxic Species (ROTS)

It is not generally known that various pathologies and the utilization of oxygen by animals leads to toxic biochemical metabolites which the body compensates partially but not completely. The byproducts of oxygen utilization are toxic and are compensated for by specific enzymes which are present solely for their destruction.

Thus, the body is protected against the toxic effects of oxygen metabolites almost completely unless the anti-oxidant mechanisms begin to wane with age, or excessive oxidative production.

The principal toxic substances referred to are superoxide and hydrogen peroxide, respectively destroyed by superoxide dismutase and catalase. A third toxic substance, hydroxyl radical, is formed by their union but is not destroyed by any enzyme and exogenous therapies must be used.

These damaging byproducts of oxygen metabolism we have elected to call Reactive Oxygen Toxic Species or ROTS, amply described in another publication of the Bradford Research Institute.[53]

One of the most damaging effects of various members of the ROTS family is the peroxidation of long-chain unsaturated fatty acids found in membrane lipids. The "unsaturations" found in fatty acids consist of carbon-carbon double bonds which are specifically placed along the chain. Peroxidation effectively converts unsaturated carbons to saturated carbons. When unsaturated fats (safflower oil, for example) are partially hydrogenated resulting in less unsaturations, the melting point is raised producing a semi-solid. As a result of peroxidation, membrane lipids become less mobile resulting in lowered membrane fluidity.[55,56,57] A decrease in membrane fluidity makes it less likely that cell recognition receptors will undergo lateral motion and become aligned with corresponding

receptors in the bound membrane of the fetal cell. A reduced binding between cell recognition receptors lessens the response to contact with fetal cell receptors.

The relationship described above between excessive ROTS and the response to live cell therapy implies that the level of ROTS should be monitored before therapy is begun. This is best accomplished through the use of the HLB Blood Test, an indicator of excessive ROTS in the blood.[53] The HLB test for ROTS is used to titrate anti-oxidant therapy to minimize excessive oxidative processes which affect membrane fluidity, receptor activity, and diminish the efficacy of live cell therapy.

AGE OF THE EMBRYO

Evidence for the significance of the age of the embryo is the observation of an age-dependent decline in the ability of dissociated embryonic chick retina and chick and mouse brain cells to reaggregate.[25,26] In the case of retina cells this loss of aggregation ability has been correlated with the loss of ability to be agglutinated with Con A (a lectin)[27] and with decreased levels of retina-specific aggregation-promoting macromolecules in the plasma membrane.[28] This age-dependency is part of the ongoing research of the Bradford Research Institute (B.R.I.) in Mexico for bovine and bovine or sheep cells.

These retina and brain neurons retain throughout their lifetime the ability to remain attached to each other. These adhesive properties of mature cells may then be different from those exhibited by dissociated embryonic cells during their initial reaggregation.[3]

These findings imply that the cell surface macromolecules (recognition receptors) present on embryonic cells are different from those of the adult. This experimental observation is a

justification for the practice of employing fetal cells of a specific age in live cell therapy.

A final implication of the significance of embryonic age in relation to live cell therapy is described in greater detail below (see Chapter III, Alpha-Fetoprotein in Live Cell Therapy). This highly significant protein peaks at 50% of term which is also the upper age limitation set by those who practice this form of therapy.

SIGNIFICANCE OF GALACTOSE

Preformed aggregates of neural retina cells were treated with various enzymes and the rate of adhesion of dissociated cells to the aggregate was measured. One result of these experiments has great implication for live cell therapy. The enzyme, beta-galactosidase (removes the sugar, galactose from carbohydrate), changed the adhesive properties of the cells so that both homotypic (cells of the same type of tissue) and heterotypic (cells of different types of tissue) cell adhesions were enhanced. There was a reduction in the specificity of contact cellular interactions, suggesting a role for a terminal galactose residue in a glycoprotein, tissue-specific recognition macromolecule. The galactose is found on the terminal ends of carbohydrate chains attached to protein.[29]

The same investigators have also demonstrated the presence of a cell surface galactosyltransferase (an enzyme that attaches galactose to carbohydrate) on dissociated retina cells and have suggested a role for this enzyme in the specific reaggregation process.[30] Evidence for the significance of this enzyme in cell aggregation is that the addition of an excess of substrates which bind to this enzyme decreased the rate of cellular adhesion.

Sialic acid (nuraminic acid) is a special modified sugar found on the terminal end of every serum protein carrying carbohydrate

with the exception of transferrin. This acid is also abundant on the surface of red blood cells and plays a major role in biological processes.[53] It has been found that the sugar immediately adjacent to sialic acid is always galactose. The enzyme sialidase (nuraminidase) selectively removes sialic acid thereby explosing a galactose residue.

It was mentioned above that when galactose is removed from the terminal position of the carbohydrate moiety of lectins the specificity of cell adhesion was lowered, permitting heterotypic cell binding.[29] Asialofetuin is a protein (fetuin) from which the terminal sialic acid groups have been removed thereby exposing galactose. This protein is capable of binding to the lectin normally present on the surface of a certain cellular slime mold (Polysphondylium pallidum) and inhibits cellular adhesion.[31]

The protein with exposed galactose (asialofetuin) is bound by the lectin surface recognition and adhesion receptors implying that this lectin will bind galactose residues on the terminal ends of carbohydrate. This observation combined with the experiment described above relating to the loss of cell adhesion specificity when galactose is removed from carbohydrate terminal ends[29] implies that if the terminal ends of carbohydrate found on glycoprotein cell adhesion receptors is galactose, greater cell binding will occur.

One technique for generating additional galactose residues on carbohydrate bearing terminal groups of sialic acid is to expose the glycoproteins (or whole cells) to the action of sialidase (nuraminidase) which selectively removes the terminal sialic acid and thereby exposes galactose residues. Producing asialo cell surface recognition receptors should lead to increased cell binding as well as increased cell response to that binding, discussed in greater detail below.

Rat hepatocytes will bind to acrylamide sheets to which have been bound galactose residues. This is in complete agreement with that stated above regarding lectins having greater binding affinity for carbohydrates with terminal galactose.[38]

THE RESPONSE TO SPECIFIC CELL ADHESION

Fibroblasts

Some of the most significant discoveries in cell recognition and adhesion have been made with cultured fibroblasts (Swiss 3T3 cells). When grown in 10% calf serum these cells cease to grow at a specific cell density ($5 \times 10^4/cm^2$) even if additional nutrients are supplied.[32] One explanation of this phenomena is that a negative signal for growth arises as a result of cell to cell contact.[33] If this is true it should be possible to produce the same effect by contacting the surface of growing cells by fragments of membranes isolated from the same type of cell. This is indeed the case with the purified plasma membrane fraction from confluent cells inhibiting the growth of a sparse culture of 3T3 cells.[34] The inhibition resembles that observed by cell contact and is not due to a toxic effect of the membranes on cells. The cells are blocked in the G_1 portion of the cell cycle.

The activity in the membranes is heat labile with heat-inactivated membranes not binding to cells.[35] The membrane-inhibited cells show decreased rates of transport of uridine (a component of RNA) and alpha-aminoisobutyric acid. The active components have been extracted from the membranes by the nonionic detergent, octylglucoside.[36] This unusual substance is a synthetic detergent made by attaching an 8-carbon saturated hydrocarbon to glucose. The hydrocarbon is soluble in fat while glucose is soluble in water, yet there are no charged groups present on the molecule (nonionic).

Addition of membranes to cells mimics the events associated with cessation of growth at confluent cell density. Molecules responsible for this effect appear to be integral membrane proteins. They are uniquely resistant to treatment by proteases and are not sensitive to any glycosidases that have been tested.[39]

Simian virus (SV-40) is a viral tool much used by researchers to study the effects of virus on cells under culture. When 3T3 cells which have been infected by SV-40 are allowed to grow increased cell contact has no effect on the rate of growth.[1]

It has been observed that treatment of normal cells with proteolytic enzymes produces an increase in the lateral mobility of lectin receptors.[40] It has been suggested that peripheral membrane proteins may be restricting the lateral mobility of intrinsic membrane proteins. A specific glycoprotein found in large amounts on the surface of normal cells is missing from transformed (cancerous) cells. Therefore, peripheral membrane proteins may indirectly influence the lectin-induced agglutination of cells by controlling the mobility and distribution of these proteins.[3]

When added to a sparse culture of 3T3 cells a surface membrane-enriched fraction from 3T3 cells inhibited the rate of DNA synthesis in a time- and concentration-dependent manner. The membrane preparation had no effect on the rate of DNA synthesis of SV-40 transformed 3T3 cells. A similar membrane preparation from transformed cells had a lesser inhibitory effect on 3T3 cells and no effect on transformed cells. These results indicate that surface membranes can mimic the effect of increasing cell density on DNA synthesis in normal cells.[41] See Fig. 5.

Retinal Cells

When plasma membranes were obtained from retinal and tectal cells of different age chick embryos they preferentially

Response of Fibroblasts to Fibroblast Membranes

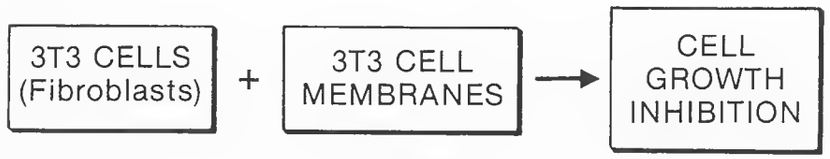


Fig. 5A

blocked the aggregation of homologous cells of the same developmental state.[37] This indicates significant and rapid changes in cell adhesion during development.

This finding has significance in live cell therapy, implying that the age of the embryo has significance in relation to the nature of cell recognition receptors found on fetal cells. The full significance of this will be discussed in greater detail below.

A low molecular weight protein has been purified to homogeneity from plasma membranes of chick neural retina. The molecular weight is 10,000 and in the presence of calcium ion the protein polymerizes to form filaments.[52] This protein blocks the aggregation of retinal cells.

Response of Virus-Infected Fibroblasts to Fibroblast Membranes

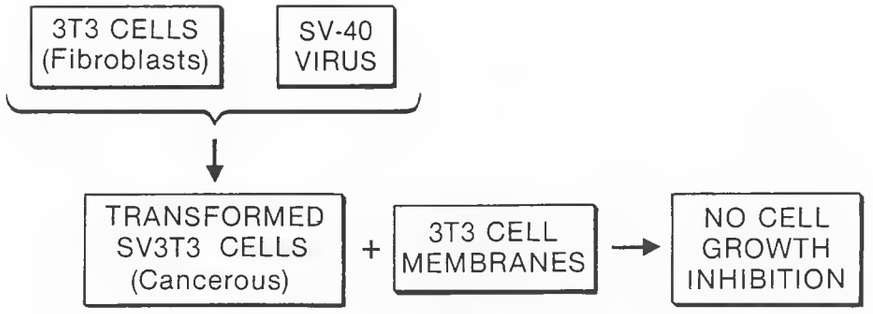


Fig. 5B

Response of Virus-Infected Fibroblasts to Transformed Fibroblast Membranes

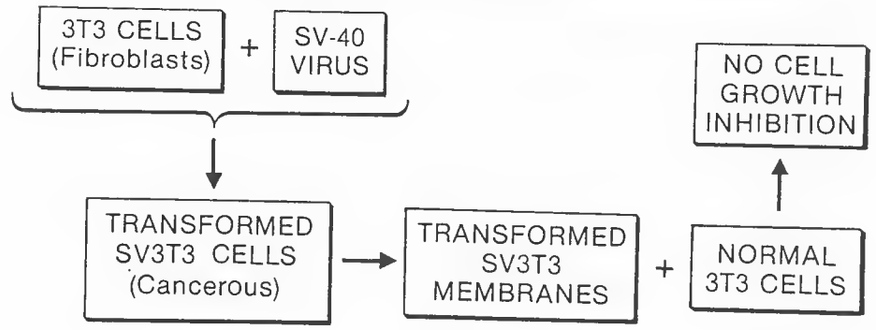


Fig. 5C

Schwann Cells

In culture, Schwann cells (a type of nerve cell) grow only in the vicinity of dorsal root ganglia neurites. It has been shown that a membrane fraction prepared from neurites acts as a very specific stimulator (mitogen) for Schwann cells. The mitogenic activity appears to be localized on the surface of the cells.[42]

In the case of both 3T3 cells and Schwann cells the binding of specific membrane protein to the cell surface has a transmembrane effect with profound metabolic consequences. Schwann cells require contact with appropriate axons present in the culture[43-45] providing a highly specific mitogen. This effect can be reproduced (as with 3T3 cells) by the addition of neurite surface membranes to Schwann cells.[4]

Certain cellular changes representing the behavioral repertoire of the cell may be induced as a result of contact cellular interactions that do not result in the formation of a stable cellular aggregate. These may include cell growth, cell division, movement, pinocytosis, secretion, biochemical composition and differentiation.[3]

In many cells under culture conditions the induction of the synthesis of differentiated proteins is partially the result of signals arising from cell contact.[2]

As a final comment on the response of cells to signals received through cell contact, it is interesting to note that plant lectins cause (1) the induction of insulin release from pancreatic islet cells, (2) the stimulation of lymphocytes to undergo mitosis, (3) mimicking of the thrombin-induced aggregation and release reaction of human platelets and (4) the inhibition of phagocytosis in granulocytes.[3]

THE IMPLICATIONS OF CELL CONTACT AND ADHESION IN LIVE CELL THERAPY

Since the time of the initial successes of live cell therapy in treating intractable diseases, conditions which would respond to no other therapy, there has been a persistent question in the minds of its practitioners, that is, "How does it work?" Despite extensive research over the years this question has remained unanswered. However, from what has been presented above there begins to emerge a clear picture of the rationale behind the success of live cell therapy. Much of the research which has been performed centered on attempting to determine the fate of the cells following injection. In answering this question researchers were confronted with an even greater enigma. It was shown that the fate of the cells, shortly after injection, was (apparently) complete dissolution to the extent that no cellular material remained visible in the optical microscope.

Inflammatory Response

It is well known that, even though the immunological response to the injection of fetal cells invokes a lesserinflammatory reaction than otherwise (possibly because of alpha-fetoprotein), if this response is too great the potential value of the treatment is highly jeopardized and may not manifest. As a result of this immunological process (inflammation at the injection site) digestive enzymes are released into the area from the rupture of lysosomes, enzymes which because of their diversity are capable of reducing each type of biological material present to its basic components, thereby rendering it worthless.

It is obvious, then, that for live cell therapy to exert a beneficial effect upon the recipient (which it is known clinically

to do) it becomes necessary for one or more basic biological structures having significance to living cells to survive this digestive process and persist.

One such candidate is DNA, the "master template" present in all cells, which contains the total information for synthesizing and defining all of the protein in the body (including enzymes). Mechanisms are known whereby this highly significant biological material may be incorporated into cellular DNA in the same manner as viral DNA, thereby potentially replacing a damaged gene in an old cell with an intact one.[46]

A second and even more promising biological structure which has direct bearing on the function and performance of all cells in the body is the cell recognition and adhesion receptor. As we have seen above these vital proteins are at the root of cellular control and respond with great specificity to appropriate external stimuli. It has also been stated that these cell receptors, composed of both protein and carbohydrate, are highly resistant to both proteases (enzymes digesting protein) and glycosidases (enzymes digesting carbohydrate). This resistance to hydrolysis by these two classes of enzymes accounts for the ability of cell recognition receptors to survive the digestive action of lysosomal enzymes released during the inflammatory process.

The degree of the inflammatory response to the intramuscular injection of live cells has significance in relation to the beneficial effect to be derived from the injection. If the inflammatory reaction should be too little there would not be sufficient liberation of recognition proteins to ellicit a beneficial response.

On the other hand, should the inflammatory response be too great the concentration of digestive enzymes in addition to the stimulation of phagocytosis may result in complete digestion of the glycoproteins of significance. It has been observed clinically that

if for any reason, there is an excessive inflammatory response to the injection, no beneficial effect is noted from the therapy.

Specificity of Binding

As mentioned above the specificity of binding fetal cellular material with human tissues lies in the high degree of affinity between cell recognition receptors of the same tissue type. It is only necessary that either membrane fragments or the membrane-bound receptors survive the initial inflammatory response. Experiments conducted in vitro with cultured cells reveals that in mixed cell populations of different species but of the same tissue, stable aggregates will form.[47,48] This implies that there are sufficient similarities between the receptors of the same tissue but different species to permit specific binding to occur. The implication for live cell therapy is that beneficial effects obtained from fetal cells of an animal (usually of sheep or bovine origin) will be obtained as a result of this similarity.

It is this similarity of cell recognition receptors which enables those receptors present on fetal cells of a given tissue type to bind to the corresponding receptors on the surface of cells of the same tissue type in the recipient. In addition, it is the presence in fetal cells of non-specific cell adhesion proteins which permit a membrane fragment from a pancreas cell, for example, to adhere to cells of another organ thereby losing the beneficial effect which would otherwise be obtained from that fragment.

Both the specific[20,21] and non-specific[7] binding of fetal cell material to tissues of the recipient have been observed in research conducted with experimental animals.

Two separate lines of research[15,37] have implicated the age of embryonic cells as being of importance in relation to the presence of cell recognition receptors. In one experiment it was shown that,

with tissues of the same type but of different embryonic age, there was a greater aggregation (binding ability) between cells of the same developmental stage (fetal age).[37] This demonstrates that there is a change during fetal development of the cell recognition receptors. There is ongoing research at the BRI in Mexico to determine the best fetal age for the greatest response to live cell therapy, perhaps also implying a specific age for each tissue type for optimum response.

Duration of Responses

The following description of the response to intramuscular injections of fetal cells is typical and does not imply that these effects are seen in every instance exactly as described.

The first response is a transient one and occurs within 24 hours following injection but may be noticed after only 4 hours. This rapid response may result from the fact that whole, intact cells are contacting the cell recognition receptors of adult muscle tissue. The cells are also bathed by adult serum possibly coming into contact with mitogens to which the cell is responding. These external stimuli may be capable of causing the synthesis of new cell surface receptors followed by an internal response resulting from contact with adult tissue receptors already present.

Also at this stage is the synthesis by the fetal cell of alpha-fetoprotein which has an inhibitory action directed towards the immunological response of the host to the injected cells.

A second transient response is noted after about 4 days. At this time no cellular material remains. This effect may arise from liberated fetal cell recognition receptors contacting receptors already present on cells of the recipient. If such receptors are either deficient or defective the response would be a basic genetic one, reaching to the DNA of the cell. Genes long dormant are

activated resulting in the synthesis of hormones, enzymes and other substances vital to normal cell functioning.

Following a period of approximately 4 months a permanent response is noted which may last for many years. As a result of contact with fetal cell receptors host cells are restored to normal activity and contribute a cyclic effect to the whole body whereby improved performance of one organ assists all other related organs.

It was indicated above that when either whole cells or isolated membrane fragments (of the same tissue) contact cells the binding is at first reversible and then becomes irreversible.[1,6] This permanent binding explains the long-term, long-lasting effects seen clinically with live cell therapy. This type of response may be exemplified by treatments given to Mongoloid children in which the noted improvement appears to be permanent with no signs of regression or relapse.

Neurological Diseases

Some of the greatest successes in live cell therapy have been made with various diseases of neurological origin, for example, Mongolism, Alzheimer's disease, multiple sclerosis and ALS. As indicated above there is a highly specific response of Schwann cells in contacting neurons. This contact triggers their growth in vitro[42-45] and undoubtedly is responsible for the favorable response to the administration of fetal nerve cells in patients having neurological diseases.

IMPLICATIONS OF CELL RECOGNITION MECHANISMS TO LIVE CELL THERAPY

An understanding of the mechanism of action of live cell therapy leads to the postulation of several modifications to the

technique as it is presently practiced as well as shedding new light on evaluating present procedures.

Nuraminidase

As indicated above, several independent studies in cell recognition and adhesion indicate that the binding affinity of lectins (specific proteins binding carbohydrate) present on membrane-bound glycoprotein receptors is increased when the terminal sugar of the carbohydrate is galactose.[29-31] It is well known that terminal sialic acid, when removed, always exposes a galactose residue. By exposing dissociated fetal cells to the action of nuraminidase, sialic acid is selectively removed thereby exposing a galactose residue. This simple process should increase the binding affinity of fetal live cell recognition receptors to the target cells of the recipient.

Unsaturated Fatty Acids — Cholesterol

It is obvious that for two opposing membranes to bind together through the specific affinity of molecular receptors imbedded in those membranes it becomes necessary for at least one of the receptors to undergo a rapid lateral motion within the membrane. The exact mechanism of this molecular displacement is unknown, however, experiments have shown that a specific cellular binding is enhanced when the fluidity of the membrane is sufficiently high.[3] The fluidity of bilayer lipid membranes is to a great extent determined by the type of lipid comprising that membrane which is, in turn, related to the degree of unsaturation in the fatty acids making up the lipids. It has been shown that the composition of membranes in relation to the degree of unsaturated fatty acids present may be modified within a relatively short period of time (2 weeks) by dietary supplementation of the desired fatty acids (for example, eicosapentaenoic acid or EPA).[49]

These realizations introduce a new concept in live cell therapy, that of "priming" the patient before therapy is begun. An improvement in membrane fluidity increases the binding affinity of fetal material and, as a result, the beneficial response to be derived from this form of treatment. The cholesterol level should also be normalized to assist in attaining a proper membrane fluidity.

Sodium Butyrate (Butyric Acid)

It was mentioned above that in several cells under culture conditions proteins that are normally (in vivo) produced only when the cells are in a highly differentiated state are seen only when cell-to-cell contact is present in the culture.[2] This observation may be coupled with the finding that sodium butyrate (butyric acid) has been shown to cause some types of cancerous cells (representing a non-differentiated state) to become more differentiated and therefore more like normal cells.[50,51] This response from sodium butyrate may result from the stimulation of the synthesis of cell recognition receptors or other peripheral proteins on the cell surface which are missing from cancerous cells.

By inference, sodium butyrate is an agent which may exert its beneficial effect on cancerous cells by stimulating the synthesis of vital membrane proteins which, through cell contact, are able to restore, at least partially, the normal metabolism of the cell. The use of this agent before cell therapy is again justification for priming the patient before treatment with the purpose of increasing the number of cell recognition receptors on the surface of cells. This, in turn, increases the binding affinity for fetal cell membrane fragments and/or cell recognition receptors resulting in a greater response to the fetal cells.

Whole Cells versus Freeze-Dried Cells

Practitioners of live cell therapy are presently employing two different methods for preparing fetal cells for injection. In one technique whole, fresh cells are quick frozen (to prevent cell rupture) and stored until used. In the second technique fresh cells are freeze-dried and stored in the frozen state as an anhydrous powder.

Freeze-drying provides a means of preserving cellular material over long periods of time as well as permitting the shipment of fetal cells under conditions where freezing is not provided for relatively short periods of time.

However, the process for preparing freeze-dried cells ruptures the cells thereby releasing antigenic material which immediately stimulates the immunological system of the recipient. With whole cell (or tissue) injections the only antigens present are those proteins on the surface of the cells. Whole fetal cells also produce large amounts of alpha-fetoprotein (described fully in Chapter III) which exerts an inhibitory effect on immunological responses to foreign material.

As mentioned above under Inflammatory Response, the magnitude of the inflammatory response resulting from the injection of cellular material may very well determine the value received from the treatment. Too great and rapid a response leads to the release of chemotactic peptides, attracting white cells (phagocytosis) and the production of antibody with the resulting formation of antigen-antibody complexes. These complexes activate the complement system which causes the eventual lysis (rupturing) of fetal cells and the release of antigenic material.

It is seen that these processes are cyclic, that is, once begun they result in a cascade (domino) effect which multiplies and intensifies the original response. As described above, if the immuno-

logical response to fetal cell injection is too great the cell recognition receptors may be destroyed through phagocytosis and excessive enzymic digestion.

Fetal Cell Preparation

While cell preparation techniques are more fully described in another chapter there will be presented here a modification to the method presently in use which may represent an advancement, particularly in relation to the concepts of cell recognition and adhesion.

Since cell recognition and adhesion receptors are found on the cell surface it would be advantageous to expose as many of these glycoproteins as possible. This may be accomplished by either of two methods.

(1) A fine mesh stainless steel screen wire (of a few thousandths of an inch mesh) is cut to a circular shape and welded into the bottom of a stainless steel surgical pan (with a hole cut in the center). Using sterile technique, tissue is gently pressed through the screen wire and scraped off the bottom. The cells are suspended in a physiological nutrient medium.

(2) A Potter homogenizer for cell dissociation consists of a Teflon pestle or rod with rounded end mounted on a 1/4" steel shaft. The steel shaft is mounted in a rotary press. The tissue to be dissociated is placed in a tight-fitting test tube especially made to have a specific clearance (a few thousandths of an inch) with the pestle. Tissue is placed in the glass tube along with a small amount of nutrient medium. The rotary press is turned on and the glass tube with the tissue is slowly brought higher and higher, forcing the tissue into the clearance between the pestle and tube, fragmenting it into individual cells. The glass tube is constantly submerged in an ice water bath to prevent heating. The

entire assembly is autoclavable and kept sterile during the operation.

Either of these techniques is for the purpose of dissociating fetal tissue to free cells. A second reason for dissociation is to expose the cell surfaces to the action of nuraminidase as described above.

THE SIGNIFICANCE OF RNA

The use of RNA as a dietary supplement has had widespread usage since being made commercially available to the general public. RNA (ribonucleic acid) is one of two nucleic acids (the other being DNA) found in both plant and animal cells. Both RNA and DNA are polymers, that is, long chains formed of several similar units (monomers) linked together in a specific manner.

There are only four different units comprising RNA, known as nucleotides. Each nucleotide consists of an organic ring (or for two nucleotides, two rings fused together) known as a "base" coupled to the 5-carbon sugar, ribose, which itself is a ring with one carbon left out of the ring. The carbon not in the ring is bound to a phosphate group. If the phosphate group is not present the unit is known as a nuceleoside.

The four kinds of nucleotides differ only in the structure of the base with both the ribose and phosphate groups identical in all nucleotides. The only difference between one RNA and another is the sequence of the four bases along the chain. See Fig. 6.

Types of RNA

There are three major types of RNA found in animal cells, namely, messenger RNA (m-RNA), ribosomal RNA (r-RNA) and transfer RNA (t-RNA). All three of these RNAs are intimately involved in protein synthesis. So that the meaning and significance

of these RNAs may be understood the activity of each will be very briefly described, omitting many details.

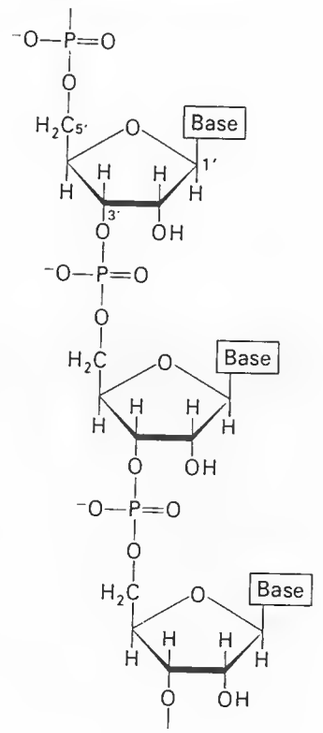


Fig. 6

Each protein is a chain of approximately 20 different kinds of amino acids linked together in a specific sequence. Here too, as with RNA, the difference between one protein and another is the sequence or arrangement of amino acids in the chain.

Mechanism of Protein Synthesis

For a cell to specify which sequence of amino acids is to be formed some basic information defining a specific arrangement or sequence of amino acids must be stored within the cell initially. The information specifying amino acid sequences is stored in coded form as messenger RNA, also a chain of four basic units (nucleotides)

as described above. Since there are only four types of nucleotides (designated for simplicity A, C, G and U) there are many more possibilities for kinds of triplets of these nucleotides than would be required to specify 20 different amino acids with a different nucleotide triplet for each. Examples of triplets are UGA, UUU, ACG, etc., with each representing a specific amino acid. The complete collection of all triplet codes corresponding to the amino acids is known as the Genetic Code (see Fig. 6).

Transfer RNA

Exactly how these triplets of RNA nucleotides specify amino acids is related to the second type of RNA, that of transfer RNA (t-RNA). Each t-RNA consists of a single strand of RNA which automatically arranges itself into the form of a cross with the ends of the strand lying close to each other at the tip of the long arm. At the hairpin turn opposite the long arm is found the nucleotide triplet representing a specific amino acid. Thus, there are essentially 20 t-RNAs, each corresponding to one of the amino acids. An enzyme binding both a specific t-RNA and the corresponding amino acid couples the t-RNA to the amino acid and falls away (see Fig. 7).

The third type of RNA, ribosomal RNA (r-RNA), forms a part of a large molecular complex known as the ribosome, consisting in part of protein, and acts as a support for the various components of protein synthesis described above.

The process of protein synthesis is similar to feeding a magnetic tape through a tape recorder. As the tape (m-RNA) is fed through it is supported and processed by the read head (ribosome). The output is what might be called a second tape made of another material, namely, protein.

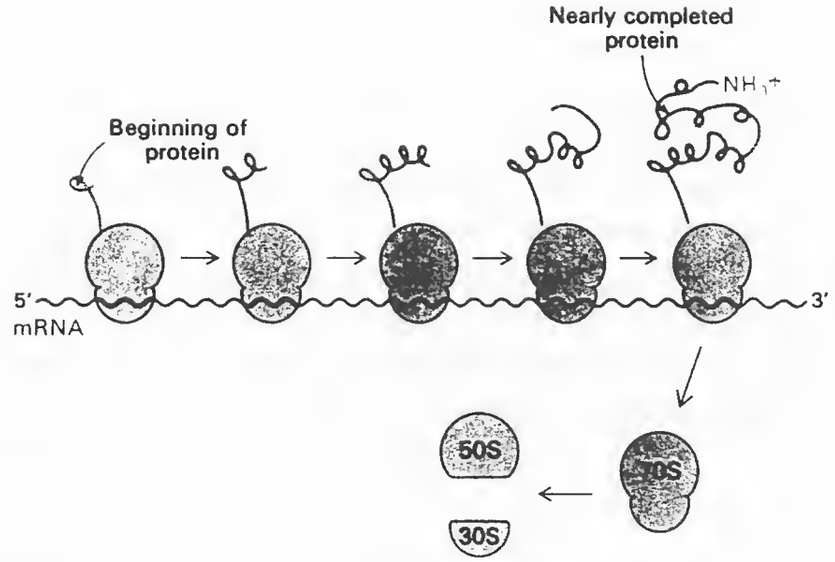


Fig. 7

In actuality, as the strand of m-RNA passes over the ribosome, each triplet of nucleotides in m-RNA binds only one type of t-RNA (cross-shaped molecule) determined by the corresponding triplet on the hairpin bend. The binding is through complimentary pairs of nucleotides (A-U or C-G) known as Codon and Anti-codon. Since each t-RNA has been bound to a specific amino acid the amino acids are permanently linked together, one after another, in the sequence defined by the nucleotide triplets found in m-RNA.

In the process the carrier t-RNA falls away as does the m-RNA, generating a strand of protein.

Lack of RNA Specificity

The mechanism of protein synthesis has been described above solely for the purpose of identifying the various types of RNA and indicating their purpose in this process. All m-RNA is chemically the same and differs only in the sequence of bases (nucleotides) making up the molecular strand. Since m-RNA (and all other RNAs as well) is biosynthesized within cells there are no receptors on the surfaces of cells that recognize RNA of any type. It is true that the m-RNA produced by a particular cell type (pancreas, for example) specifies a protein needed by pancreas cells but it is also true that, if the m-RNA for that protein were injected into the blood, it would have no way of recognizing the pancreas or entering pancreas cells or any other cell of specific type.

Because of the foregoing any claims made that m-RNA isolated from a particular organ of an animal and injected into the blood results in a specific change in the corresponding organ is unjustified on a scientific basis. The RNA available as a dietary supplement has most commonly been isolated from yeast. Yeast RNA has value to a yeast cell because it produces yeast protein but has no value (in relation to protein synthesis) to a human.

Transfer RNA from both plants and animals is very similar and, once again, differs only in the sequence of nucleotides of which it is composed. This substance should be looked upon in the same manner as a mixture of free amino acids or sugars. Since t-RNA has value only within cells where protein synthesis occurs, injecting t-RNA into the blood will not result in increased amounts within cells since there are no active transport systems for carrying this molecule across the outer cell (plasma) membrane. Orally

ingested RNA will be quite rapidly hydrolysed (broken into its constituents) by stomach acids.

Ribosomal RNA

The third type of RNA, ribosomal (r-RNA), forms a part of a large composite (the ribosome) normally attached to a subcellular membrane (the endoplasmic reticulum, ER). This composite, formed of many subunits, has value to a cell only when intact, functional and within the cell bound to the ER. The size of this unit prevents it from passing through the plasma membrane and entering a cell. If ribosomal RNA is separated from the associated protein the resulting product is not functional and cannot carry out protein synthesis. Thus, ribosomal RNA in any form (with or without the associated protein) cannot be of value to cellular metabolism per se even if injected into the blood.

Nutrient Value of RNA

In light of what has been presented above, RNA, either taken orally or by injection, would be of no value to cellular metabolism. Yet, there are many published reports describing the beneficial effects of RNA. Many people have taken RNA orally and received benefit from it. This paradoxical situation may be resolved by realizing that RNA, administered either orally or by injection, has value as a nutrient. Research has shown[54] that when the free bases of RNA are fed they are not incorporated into cellular nucleic acid but that nucleotides, when ingested, <u>are</u> incorporated. Also, when whole RNA is fed the nucleotides are incorporated because of the breakdown (hydrolysis) to these units by stomach acids.

Thus it is seen that the beneficial effects of administering RNA is as a nutrient. This realization explains how humans benefit from yeast RNA because the units (nucleotides) are identical in

yeast and humans. Injected RNA is quickly degraded to nucleotides through the action of the enzyme, ribonuclease, present normally in the blood.

A part of the value of live fetal cell injections lies in the RNA content. Histochemical staining reveals that the concentration of RNA in fetal cells is much higher than that of the corresponding adult cells. This observation is to be expected considering that the rate of protein synthesis in rapidly growing cells is larger than that of a resting cell.

IMPLICATIONS OF CELL RECOGNITION TO CANCER THERAPY

The research described above with 3T3 cells in relation to SV-40 transformed cells (cancerous) has highly significant implications in cancer therapy. Through sufficient cell contact with similar cells DNA synthesis and cell replication is inhibited in normal 3T3 cells.[32] With virally transformed cells DNA replication and cell division is not inhibited by contact with cell recognition receptors.[1] Additional research has shown that either the receptors or other vital membrane proteins are missing from the cell surface.[3] There are apparently two requirements for inhibiting cell division, (1) the presence on the cell surface of certain critical proteins and (2) contact with cell recognition receptors by specific lectins found in membranes of the same cell type.

A substance stimulating the production of cell surface membrane proteins may be sodium butyrate. The first requirement in cancer therapy would be to cause the appearance on the cell surface of the appropriate membrane-bound proteins. The second requirement is satisfied by live cell therapy which provides appropriate

lectins to contact cell surface receptors thereby inhibiting DNA synthesis and cell replication.

Practitioners of live cell therapy have noted that cancer treated with injections of the same cell type may respond remarkably well. This favorable response is an indication that if the first requirement could more certainly be satisfied, cancer may quite possibly be brought under control through live cell therapy.

SUMMARY

A biochemical explanation of live cell therapy is presented which confirms results seen clinically. The long-term effects of live cell therapy result from a specific binding between cell recognition receptors present on the surfaces of fetal cells of a given organ with matching receptors on the surface of cells of the corresponding organ in the recipient. This contact may occur with whole fetal cells, membrane fragments of these cells (containing recognition receptors) or simply with the non-membrane bound receptors themselves. The result is a turn-on of cellular activity which may reach to the genes themselves.

Evidence is given for:

(1) The specificity of fetal cell binding to the corresponding organ of the recipient.

(2) The non-specific binding to other organs.

(3) The significance of using fetal cells rather than adult cells.

(4) The significance of the age of the embryo.

(5) The transient first responses to live cell injections.

(6) The significance of the inflammatory response to live cell injection in relation to the success of the treatment.

(7) The long-term effect.

(8) The favorable response of neurological diseases to live cell therapy.

(9) The significance of reactive oxygen toxic species to live cell response.

Based on the biochemistry of live cell therapy as presented, suggestions for modifications to the technique as commonly practiced are given.

The significance of RNA is discussed.

The implications of these biochemical concepts to cancer therapy are presented and discussed.

CHAPTER II — PART II

Phosphorylation and Cell Surface Receptor Activity

A. PROTEIN KINASE AS A REGULATOR OF PHOSPHORYLATION

Introduction

Everyone is familiar with the amazing rapidity with which cells in distant parts of the body may communicate through nerve conduction. This process shows some similarity to the conduction of electricity through wires and is almost instantaneous.

Not so well known is a slower form of cellular communication through the agency of specific chemicals. The list of such chemicals was once comprised only of hormones, those simple organic molecules first shown to be the products of ductless or endocrine glands and secreted directly into the blood to perform their task elsewhere in the body.

Recent research has revealed that the list of chemical mediators of biological signaling is much more extensive than previously imagined. The well known hormones, for example, thyroxine, epinephrine (adrenalin), vasopressin, to mention only a few, are simple organic structures having comparatively low molecular weights. However, some of the more recent additions to this class of "effectors" or "mediators of cellular communication" have much larger molecular weights of several thousands. These newly discovered substances are polypeptides or miniproteins, molecular threads formed of short lengths of amino acids. Those that are "heat stable" have no specific self-association that is denatured or changed by heating.

Both the more recently discovered peptides and the well known hormones reveal their presence by interacting with and binding to specific surface receptors which are an integral part of the outer membrane of cells. Each such receptor is highly specific for a particular hormone or protein and will bind no others. The specificity of binding is made possible because the receptor consists of protein having a cavity on this surface lined with amino acids which interact with only the one substance for which it is made. This interaction may include "hydrogen bonds," an association between methyl groups ($-CH_3$) or between aromatic (benzene type) rings found in both the cavity and the hormone.

The outer or plasma membrane of a cell consists of lipids (fats) which are immiscible with water (hydrophobic). Because of this many large water soluble substances found outside a cell cannot pass this barricade and enter the cell. For this reason cells have developed a method of responding to the external environment without it being necessary for the signaling agents to actually enter.

When a hormone or other signaling factor (known as a "first messenger") specifically binds to its receptor, the receptor changes shape or "conformation." Most if not all receptors extend completely through the membrane, having parts which are exposed both outside and inside the cell. The shape change makes possible a reversible chemical reaction of one kind or another to occur within the cell, either on the receptor itself or in relation to an associated molecule. Thus, as a result of a first messenger binding to its receptor a signal is sent into the cell giving instructions to carry out a specific task, quite often reaching to the very heart of the cell, the genetic material within the nucleus. Through this process the cell receives instructions in response to changes which may be occurring in other parts of the body. Without the ability to respond properly to such signals, disease is the result.

Examples of first messengers are insulin, epinephrin platelet derived growth factor and epidermal growth factor, each being bound by its specific cell surface receptor.

Still another receptor (to be discussed in Chapter II, Part VI) is one found in all nerve terminals, binding the specific protein synapsin I. This receptor is highly significant in relation to live cell therapy in the treatment of neurological diseases. For the most part we are concerned in this chapter with the first messenger, epidermal growth factor (EGF) and its receptor (EGF-R) which has received considerable attention from researchers over the past few years. The reasons why so much interest has been directed toward this particular receptor-effector system and its significance in live cell therapy is described below.

To enable the reader to understand more fully the relationship between live cell therapy and the biochemical results arising from this form of therapy there follows a detailed description of the interaction between certain cell surface receptors and the biochemical responses which they elicit.

This discussion will enable the reader to understand, in relation to live cell therapy:

- Why excessive Reactive Oxygen Toxic Species (ROTS) result in a diminished response and the importance of adjunctive antioxidant therapy.
- Why chelation therapy with EDTA plus certain mineral requirements leads to an increased response.
- Why liver and the inner placenta alone rather than whole placenta should be given to cancer patients.
- Why the outer placenta has a greater effect than whole placenta on heart patients.
- Why fetal liver cells should be given to cancer patients.

- Why the skin is improved from fetal cell injections.
- Why both pre- and post-therapy diet supplementation is important.
- The implications and value in cancer therapy.
- Why some neurological diseases respond favorably to fetal neurological tissue.
- How the genetic activity of the patient's cells may be modified through incorporation of fetal cell outer membranes.

Epidermal Growth Factor

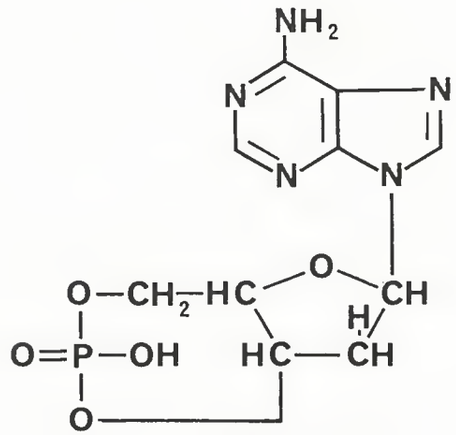
In Part I of this chapter the significance of a particular class of cell surface receptors was described and related to live cell therapy. Another membrane component of equal importance is the Epidermal Growth Factor Receptor (EGF-R) also found in fetal tissue. The binding of EGF by its receptor results in a cascade of cellular events which may eventually result in modification to the genetic material (DNA) of the cell. EGF-R is found on the outer surface of many types of adult cells and serves to regulate cellular activity. As we shall see below, this mechanism of cellular regulation and control may become defective from several standpoints as a result of aging and disease, the latter including cancer. One purpose of live cell therapy is to replace defective or absent membrane receptors with those of fetal cells. In the developing fetus these receptors are and must be effective transducers of hormonal signals generated to induce both cell proliferation and differentiation.

The response of a cell to EGF bound to its receptor involves the highly significant enzyme protein kinase (PK) as well as its counter-enzyme, phosphoprotein phosphatase (PP). The significance

of these regulatory enzymes to the cell and to live cell therapy is described below.

Cyclic AMP

With the realization that hormones exert their effects on cells by binding to specific receptors on the outer membrane, biologists were interested in learning the mechanism of action. As a part of this work it was learned that an unusual molecule known as cyclic AMP (cAMP) was somehow involved. cAMP is actually a chemically modified form of one component of the nucleic acid RNA, instrumental in protein synthesis. However, cAMP per se has no functional relationship to RNA and is a biological example of using a modification of an existing structure for an entirely different purpose. cAMP results from the nucleotide AMP (adenoside monophosphate), losing one molecule of water as the acidic phosphate group reacts with a hydroxy group (-OH) found on the ribose (sugar) component (see Fig. 8).



c-AMP
Fig. 8

As the result of hormonal interaction with its receptor an increase in cAMP results. This realization led researchers to believe that in hormonal action there is a chain of events, starting with the hormone (first messenger) and passing the information that it is present expressed as a rise in the level of cAMP (second messenger). cAMP then acts on still a third substance and so on until the expected biological effect(s) are produced.

If this concept is correct it should be possible to obtain the cellular response to the binding of a hormone solely from an increase in the level of cAMP. This is indeed the case, with administration of cAMP to a specific cell type yielding the same result as administration of the hormone related to that cell type.

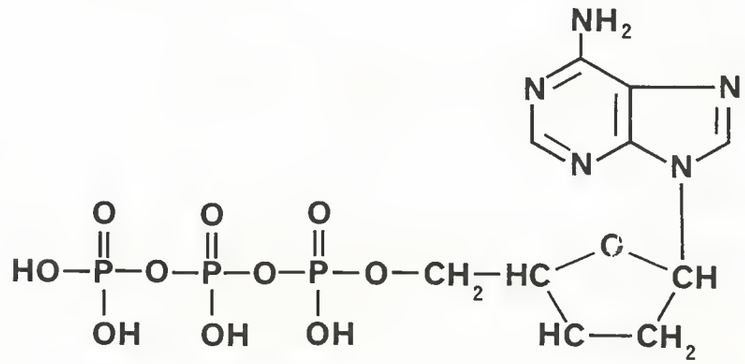
Definition of Protein Kinase

The next step in tracing the sequence of hormonal response is to determine the mode of action of cAMP. The answer to this question is extremely simple. cAMP binds to the enzyme protein kinase, having the activity of attaching a phosphate group to specific cellular proteins (protein phosphorylation). As has been determined over the past few years the regulation of almost all cellular activity is controlled by this process. Almost all of the biological effects of cAMP are mediated through the expression of PK.[58]

There are two forms (isoenzymes) of PK designated type I and type II. Both types I and II are composed of two regulatory subunits (R) and two catalytic subunits (C) designated R_2C_2. The regulatory subunits are different in the two types and are designated R_I and R_{II}. The catalytic subunits for both types are the same and are simply designated, C. It has been shown that two molecules of cAMP bind to each R subunit resulting in dissociation of the two C subunits according to the equation

$$R_2C_2 \text{ (inactive)} + 4 \text{ cAMP} \rightleftharpoons R\begin{pmatrix}\text{cAMP}\\ \text{cAMP}\end{pmatrix}_2 + 2 \text{ C (active)}$$

Only the free form of the catalytic subunit is able to phosphorylate proteins. Type I protein kinase is activated at a lower concentration of cAMP than is the type II kinase.[59]



ATP
Fig. 9

Adenosine monophosphate (AMP) bound to two additional phosphate groups forms adenosine triphosphate (ATP) in which the three phosphate groups form a chain (see Fig. 9). This compound is the actual phosphorylating agent while PK acts not only as a carrier but gives specificity to protein phosphorylation. Not all proteins coming into contact with PK are phosphorylated and, as we shall see below, only those proteins having a specific sequence of amino acids over a very short length are substrates for PK.

The ATP binding site is found on the catalytic subunit and it is this ability of C that enables it to be active in phosphorylation. ATP must be in the form of the magnesium salt for binding to occur.[60] (See Fig. 10.)

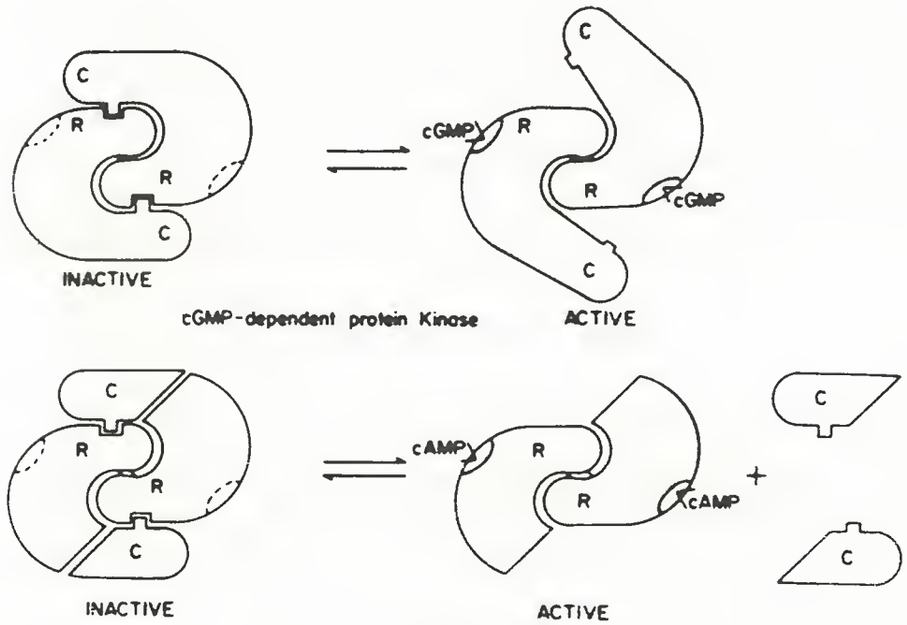


Fig. 10

Substrates of Protein Kinase

The following partial list of cellular proteins phosphorylated by PK indicates the significance of this enzyme in cell regulation and includes microtubule-associated proteins,[61] ribosomal S6 protein,[62] myosin light chain,[63] initiation factor 2 for protein synthesis[64] and nuclear proteins (histones).[65] The histones are basic proteins which wrap around DNA thereby controlling its activity. It becomes obvious that the phosphorylation of these masks of DNA

is an extremely critical process intimately related to cellular activity and various enzymes by an alteration of genetic transcription.[66]

Not all of the amino acid residues comprising protein are capable of phosphorylation. By analyzing the amino acids of phosphorylated proteins it was found that those amino acid residues carrying hydroxy groups (-OH) were the only ones phosphorylated. These amino acids are serine (R = CH_2OH), threonine (R = $CH(OH)(CH_3)$) and tyrosine (R = $CH_2C_6H_4OH$). As an example, phosphorylated serine is shown in Fig. 11.

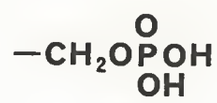


Fig. 11

If there were no substrate specificity or selection operating in relation to the activity of PK, all accessible amino acids having hydroxy groups would be phosphorylated. In analyzing the amino acid sequences near the phosphorylation site a pattern of amino acid sequence emerged which revealed the specificity requirement for PK. Although some variation from this sequence is permitted, the most commonly found amino acid sequence is Arg-Arg-X-Ser where Arg = arginine, Ser = serine (the phosphorylation site) and X = almost any amino acid. The nonspecific amino acid X represents a spacer which apparently is necessary for PK activity.[67]

The amino acid sequence of a natural peptide inhibitor of PK was determined. The active part, which included the sequence Arg-Arg-X-Ala, conforms to the pattern of a PK substrate except

for the substitution of Ala (alanine) for serine. The structure of alanine is that of serine with the hydroxy group (-OH) replaced by H. Because of the absence of a hydroxy group, alanine cannot be phosphorylated yet the other requirements for a PK substrate are fulfilled. Because of these considerations a peptide containing the sequence, Arg-Arg-X-Ala, will be bound by PK but, because it cannot be phosphorylated, will continue to be bound, block the active site and function as an inhibitor.[67]

Evidence has been presented showing that both the R and C subunits are transported to the nuclear membrane, pass through, enter the nucleus and bind to chromatin.[60,68] More will be said of these findings below in relation to live cell therapy.

Direct injection into neurons has shown that the C subunit can regulate closing of potassium ion channels.[69] In addition, the C subunit can increase the slow inward calcium ion current into cardiac myocytes.[70]

Protein kinase C is a cAMP-independent enzyme first discovered in 1977 and is found in all tissues and organs of mammals and other higher organisms.[71] It is not to be confused with the C subunit of cAMP-dependent protein kinase described above. This enzyme is calcium activated and dependent upon phospholipids for activity. Glycerol (glycerine) is a 3-carbon chain carrying a hydroxy group (-OH) on each carbon (see Fig. 12). Since these are alcohol groups they may form esters with carboxylic acids. If two fatty acids form esters with two adjacent hydroxy groups of glycerol the resultant molecule is known as a diacylglycerol(see Fig. 13). Phosphorylation of the remaining hydroxy group leads to a phospholipid.

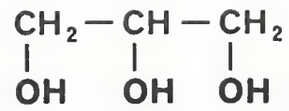


Fig. 12

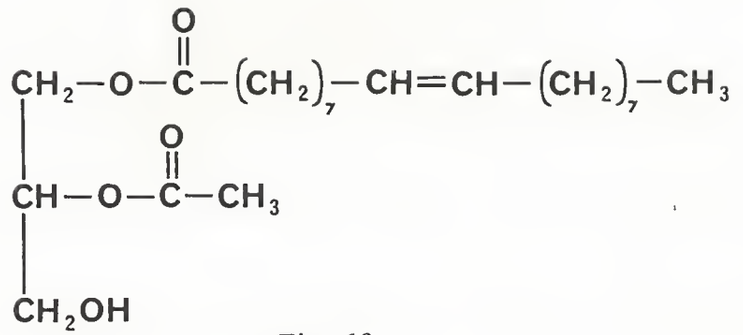


Fig. 13

Inositol is a 6-carbon ring with each carbon carrying a hydroxy group and in this respect shows some similarity to the 6-carbon sugars (hexoses). Inositol phospholipid is formed by linking inositol through one of its hydroxy groups to the phosphate group of phospholipid (see Fig. 14) where I represents inositol, P represents phosphate and R_I, R_{II}, fatty acids.

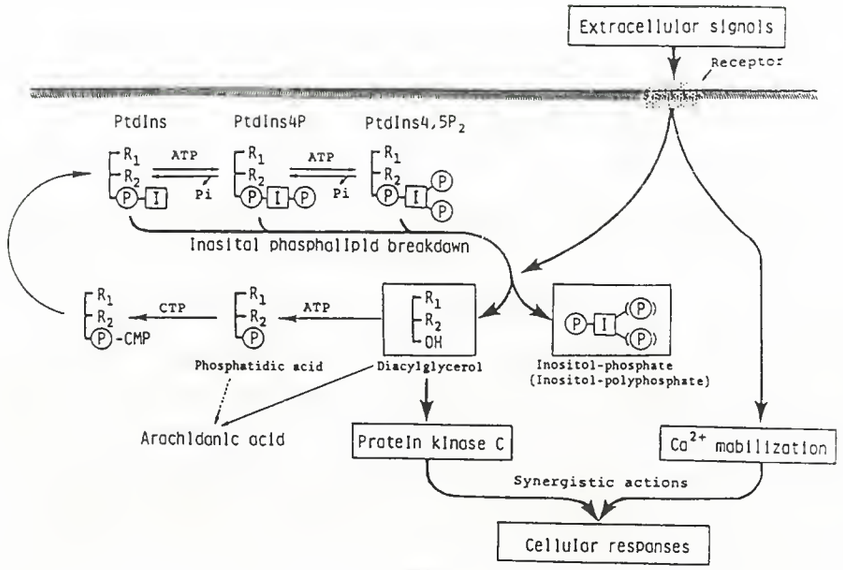


Fig. 14

When inositolphospholipid is further phosphorylated by the addition of two additional phosphate groups, phosphatidylinositol biphosphate is formed (see Fig. 14).

An extracellular signal (hormone, growth factor, etc.) binding to its specific receptor causes the breakdown of phosphatidylinositol biphosphate to diacylglycerol and inositol polyphosphate (IP_3). Diacylglycerol activates protein kinase C by greatly increasing its affinity for calcium.

The diaclglycerol so formed is phosphorylated (by ATP) and subsequently bound once again to inositol. Thus, diacylglycerol is rapidly recycled and only transiently produced in membranes.[72]

Inositol polyphosphate has been shown to stimulate protein kinase C as well as cause the release of calcium from intracellular stores.[73]

Inhibitors of Protein Kinase

The C subunit of cAMP-dependent PK has a sulfhydryl group (-SH) at the active site which reacts with a specific chemical (N-tosyl-L-lysine chloromethyl ketone) thereby inactivating the enzyme. This reaction shows that a sulfhydryl group is found near the active site but does not prove direct involvement in the catalytic mechanism since thiocyanolation of the sulfhydryl groups does not affect the catalytic properties of the enzyme.[58]

Several antipsychotic drugs (chlorpromazine, imipramine and others) inhibit protein kinase C. This response may indicate that the presence of a psychotic state is in some way related to excessive protein phosphorylation. Protein kinase C is also inhibited by vitamin E.[71]

It has been demonstrated that the protein which appears after malignant transformation by both avian (bird) sarcoma virus and leukemia virus is associated with cAMP-independent protein kinase C activity.[74] The activity of this type of kinase is inhibited by the bioflavonoid quercetin. When the well known dietary supplement rutin is hydrolysed (by stomach acids, for example) the product is quercetin.[75]

Characteristics of Protein Kinase Types I and II

cAMP-dependent PK is found in two forms designated type I and type II. Type II can phosphorylate itself (autophosphorylation)

and reassociation may require dephosphorylation. Generally, embryonic tissues and other relatively undifferentiated tissues contain type II almost exclusively.[66]

Recent research suggests different biological roles for type I and type II protein kinase. Testes from fetal and 2-day old rats exhibited only type I PK activity while type II levels increased gradually during the first postnatal weeks, reaching maximal levels at day 25. Alterations of type II PK occurred at a time that coincided with differentiation. These results suggest that type II PK may be involved in the process of differentiation which is known to respond to cAMP levels.[66]

The appearance of type I PK is seen in 3T3 cells (fibroblasts) following viral transformation.[76] Virus-transformed cell cytosols contained both type I and type II while cytosol from normal 3T3 cells contained only type II. These results suggest that type I and type II PK have different functions in the processes of growth and differentiation.[66] Transformed cells represent a form of dedifferentiation.

In experimental cardiac hypertrophy (isoproterenol-induced) there was noted an increase in type I PK. By day 10 there was nearly a twofold increase in the amount of type I PK in rat heart. Futhermore, after cessation of drug administration, PK returned to control levels. The amount of type II PK did not change significantly during the entire period of observation.[66]

These results suggest that type I cAMP-dependent PK, which has been implicated in the regulation of several heart functions including the mediation of ionotropic and chronotropic responses and glycogenolysis as well, may also be involved in the regulation of RNA and protein synthesis by mediating specific gene expression and enzyme induction.[66]

The indicated activities of cAMP-dependent PK have significant implications for live cell therapy in relation to the administration of the inner and outer placenta rather than a whole placenta preparation to heart and cancer patients. These aspects will be discussed in greater detail below under the major heading, Cell Regulation Through Epidermal Growth Factor Receptors.

In Friend erythroleukemia cells induction of differentiation by 2% DMSO was shown to result in a threefold increase in the regulator subunit of type II PK (R_{II}), a threefold decrease in R_I and an R_{II}/R_I ratio of 11 compared to 1.2 in control, untreated cells.[77] Furthermore, DMSO treatment produced an increase in C subunit activity. Moreover, the R-C subunits were present as intact tetramers (R bound to C).[59]

Another study of differentiation induced by injections of estradiol (estrogen) showed a 10- to 20-fold increase in the R_{II} content of granulosa cells.[78]

Significance of Protein Kinase in Cancer

It has been noted that a higher phosphorylating capacity is found in hepatoma cells than in normal liver. This is, once again, an example of excessive phosphorylation associated with a disease state.[79]

A well known and greatly used research tool is the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA), isolated from croton oil. It was mentioned previously that diacylglycerol greatly increases the affinity of protein kinase C for calcium, thereby activating the enzyme.

It has been noted from the structure of TPA that it (effectively) contains the diacylglycerol structure along with several fused polycyclic rings (see Fig. 15). Like diacylglycerol, TPA also

activates PK-C in the same manner. It has been shown that synthetic diacylglycerols with acetic acid substituted for one of the long-chain fatty acids are fully capable of stimulating PK-C activity in relation to calcium affinity. From this evidence it is believed that PK-C is a receptor of tumor promotors[72] and keep the enzyme always active.[71] If this is true it is still another example of excessive phosphorylation leading to a disease state. The significance of this realization will be discussed more fully below in relation to live cell therapy.

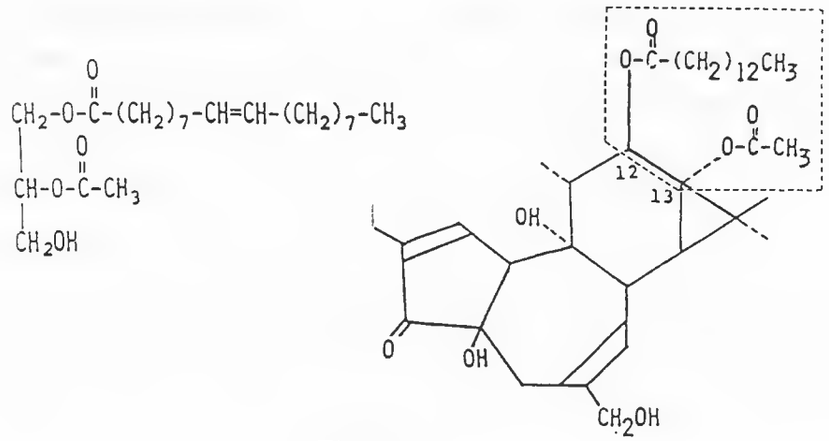


Fig. 15

Sensitivity of cAMP-Dependent Protein Kinase to Salt Concentration

It may be shown that the activity of the two sulfhydryl groups of cAMP-dependent PK are sensitive to the concentration of salt, this sensitivity being caused by a conformational change in the enzyme. Upon increasing the ionic strength of the medium from lower to greater than physiological values, one sulfhydryl group becomes more reactive while the other becomes less reactive to a specific sulfur-containing reagent. Modification of only one of the sulfhydryl groups causes inactivation of the enzyme. It has been shown that this group is related to the ATP binding site of the C subunit.[80]

As indicated above, cAMP is required for activation of cAMP-dependent PK. cAMP is synthesized by the enzyme, adenylate cyclase, which has been shown to be activated by the mineral vanadium (vanadate).[81] More will be said below about the significance of this little known dietary factor.

X-ray crystal analysis is a lengthy technique for determining the complete structure, at the atomic level, of large molecules including enzymes. The X-ray analysis of the C subunit of PK is presently being conducted at the University of California, San Diego.[82] A similar analysis of the R subunit is being conducted at the University of Illinois.[83] In the near future the complete structure of this highly significant enzyme will be known.

B. PHOSPHOPROTEIN PHOSPHATASE AS A REGULATOR OF DEPHOSPHORYLATION

Introduction

The activity of an enzyme which binds phosphate groups to specific amino acids (in protein) was described above. It is a basic biological principle that for every enzyme performing a specific task there is an opposing counter force which maintains a balance. The enzyme balancing the activity of protein kinase is known as phosphoprotein phosphatase (PP). This enzyme removes phosphate groups from protein (dephosphorylation).

Much of cell regulation carried out by PP (as well as protein kinase) is modulated by various inhibitors and activators. As will be shown below there is a cyclic interaction between inhibitors and activators of both enzymes. The presence of a phosphate group on an inhibitor may activate this agent which in turn, is regulated by the enzyme (PP) which may remove that phosphate group. Many of these regulating and controlling mechanisms which are just now being discovered, are giving us additional insight into live cell as well as pre-and post-adjunctive therapies.

Definition and Specificity

Bovine brain PP consists of a catalytic subunit A (molecular weight 60,000) and a regulatory subunit B (molecular weight 19,000). The catalytic subunit A requires manganese for activity and may bind this metal permanently. The regulatory subunit B binds four calcium ions with a high affinity and is catalytically inactive. This enzyme is present in high concentrations in the brain during the formation of synapses.[84]

Protein kinase phosphorylates only serine and threonine residues. However, a kinase associated with the epidermal growth factor receptor (to be discussed fully in Part III) phosphorylates tyrosine residues.

In chick brain extracts it has been shown that the phosphoprotein phosphatases that dephosphorylate tyrosine residues are different from those that dephosphorylate serine/threonine residues.[85]

Analysis of skeletal and cardiac muscle has shown that there are three basic categories of PP classed according to their substrate specificities. One type has a high preference for histones. It was also shown that even though the regulatory subunits differ the catalytic subunit of all three types may be identical and have a molecular weight of 35,000.[86]

Inhibitors

Two different types of peptide (protein) inhibitors have been isolated from rabbit skeletal muscle designated inhibitor 1 and inhibitor 2. Inhibitor 1 required phosphorylation by cAMP-dependent protein kinase for activity while inhibitor 2 was spontaneously active.[86] If PP inhibitor 1 is phosphorylated by protein kinase the net effect is that any phosphorylation performed by PK will have a tendency to remain because the enzyme that is capable of removing them is inhibited.[86]

Sodium fluoride (or simply fluoride ion) inhibits some phosphoprotein phosphatases by about 80% but others by only 10%. PP is inhibited by fluoride but the inhibition may be removed by manganese.[86] Anyone who wishes to use a fluoridated toothpaste or drink fluoridated water may at least partly counteract the effect by dietary supplementation with manganese.

This finding may be of significance as a preventative in relation to the viral disease, AIDS. Statistics show that the cities having the highest incidence of this disease are fluoridating the water.[175]

A very intriguing discovery having many implications is that epinephrine (adrenalin) induces the phosphorylation (and activation) of PP inhibitor 1.[86] This represents a direct link between psychological states and the regulation of a broad scope of cellular activities through phosphorylation. This realization is of particular importance in relation to high levels of PP in the brain. This situation could lead to a viscous cycle wherein excessive production of epinephrine inhibits the dephosphorylation of brain proteins causing erratic behavior and a subsequent rise in epinephrine.

As a result of oxygen metabolism several highly damaging derivatives of oxygen are generated. The body is equipped through special enzymes (superoxide dismutase and catalase) to destroy most of these substances.[87] Others are destroyed by dietary factors, for example, vitamins A, C, and E to mention only a few. One of these damaging substances is hydrogen peroxide, H_2O_2, destroyed by catalase and the enzyme, glutathione peroxidase (in conjunction with glutathione). The central amino acid of glutathione is cysteine having the grouping, $-CH_2SH$ as a residue. Thus, glutathione may be represented by the symbol GSH where G represents the remainder of the molecule and SH the active portion of cysteine.[87]

The formula representing the reaction of hydrogen peroxide with glutathione is

$$2\ GSH + H_2O_2 \longrightarrow GSSG + 2\ H_2O$$

The two acidic hydrogen found on glutathione react with the extra oxygen of peroxide forming water and the so-called "oxidized"

form of glutathione, GSSG. The oxidized form of glutathione has been found to inhibit PP.[86] This inhibition may be reversed by glutathione (now commercially available as a dietary supplement). This inhibition arises from the splitting of GSSG and the formation of a mixed disulfide between GS- and a critical cysteine residue in PP thereby forming (PP)S-SG. This linkage (and inhibition) may be broken by glutathione (GSH).

This discovery is of general significance in degenerative diseases, particularly cancer. In all degenerative diseases there is an increase in reactive oxygen toxic species or ROTS including hydrogen peroxide.[87] It is also known that excessive phosphorylation is associated with the cancerous state. This observation may be explained on the basis of the inhibition of PP, the dephosphorylation enzyme, by excessive hydrogen peroxide. The inhibition of this enzyme may also lead to many viscous cycles considering the far-reaching significance of phosphorylation in cellular activity.

Activators

Two peptide activators of PP designated activators I and II were isolated from rabbit red blood cells. Activator I stimulates PP activity from 75-100% when either the 40S ribosomal subunits or phosphohistones are used as substrates. Activator II stimulates the dephosphorylation of peptide eukaryotic initiation factor 2(eIF-2). The molecular weight of factors I and II are 12,500 and 17,400, respectively.[88]

A unique second messenger-type regulator of phosphorylation has been reported which inhibits both adenylate cyclase (synthesizes cAMP) and cAMP-dependent PK as well as activating PP. The net result is a stimulation of dephosphorylation.

The regulator is believed to be prostaglandin E_1 coupled to phosphoinositol phosphate. A molecule having this structure could be formed from an ester between the long-chain fatty acid, arachidonic acid, the precursor of prostaglandins and inositol phosphate. Arachidonate cyclooxygenase could then convert arachidonic acid to prostaglandin E_1 forming the phosphorylation regulator.[89,90]

It has been learned by practitioners of live cell therapy that the concurrent administration of chelation therapy with EDTA increases the response received from cellular injections. This effect may possibly be explained in relation to certain experiments performed with PP and EDTA. Much evidence indicates that type I PP contains a tightly bound manganese ion required for activity.[86] The manganese in PP is actually chelated (held) by the organic groups surrounding it. The presence of EDTA for short periods of time failed to inactivate the enzyme while other smaller molecules (pyrophosphoryl compounds such as POP) could inactivate PP because of their ability to enter the active site and remove manganese.

However, exposure of PP to EDTA over many hours resulted in the gradual removal of manganese and inactivation of the enzyme. When reactivated with cobalt the enzymatic activity was from 2 to 3 times greater than when activated by manganese. When fully reactivated by cobalt subsequent exposure to manganese did not, however, decrease the activity to the level of the manganese-reactivated enzyme.[86]

It has been pointed out in several instances that a state of excessive phosphorylation has been associated with disease. As will be fully described in Part III the administration of live cells supplies various membrane-bound receptors to the outer surface of the patient's cells. When later stimulated by natural factors (for example, epidermal growth factor, insulin, etc.) protein kinase, a

phosphorylating agent, will be activated. Unless there is the capability to balance this augmented phosphorylation process (by PP) the balance will, at least temporarily, swing towards excessive phosphorylation. If EDTA administration accompanies live cell injection, this provides an excellent opportunity to swing the balance towards a state of less phosphorylation by withdrawing any manganese supplementation both before and after treatment followed by cobalt supplementation immediately following treatment.

Foods high in manganese (to be temporarily avoided) are bran and orange marmalade while a food high in cobalt is the mushroom.

The amino acid, tryptophan, increased the activity of rat liver phosphoprotein phosphatase.[91]

Additional Biochemical Activities

Glucocorticoid receptors in rat liver are inactivated (unable to bind steroid) by incubation with PP. Receptors that are inactivated (dephosphorylated) in the presence of molybdate can be reactivated by sulfhydryl compounds, for example, the amino acid, cysteine. However, those receptors that are inactivated in the absence of molybdate cannot be reactivated. Dephosphorylation apparently leads to oxidation of a critical sulfhydryl group that is required for steroid binding. Molybdate may act by complexing with sulfhydryl groups on the receptor.[92] The bean family, including peanuts, is an excellent dietary source of molybdinum.

Calcineurin is a specific calcium-dependent PP found in the brain consisting of two subunits designated A and B having molecular weights of 60,000 and 15,000, respectively. The amino end of the B subunit has been found to be bound to myristic acid, a 14-carbon saturated fatty acid. It is believed that the function of myristic

acid is to enable calcineurin to bind to membranes or to enable it to pass through the nuclear membrane and enter the nucleus.[93,94] An excellent dietary source of myristic acid is pollen.

The activity of type I PP towards the dephosphorylation of histones is partly dependent on cellular salt concentration[86] as is the activity of protein kinase.[80] It is well known that in cancerous cells the sodium/potassium exchange mechanism is defective and that salt concentrations are imbalanced. The influence of salt concentrations on the activity of both phosphorylating and dephosphorylating enzyme systems may be related to the transformation of normal cells to cancerous considering the extreme significance of the phosphorylation of cellular proteins in cell regulation.

Much evidence has accumulated indicating that protein phosphorylation of serine and threonine residues may act as a general physiological mechanism for mediating the effects of many classes of physiological stimuli. The Rous sarcoma virus generates, through the expression of its genetic material (in conjunction with the mechanism of protein synthesis) a protein kinase capable of phosphorylating tyrosine residues rather than serine or threonine. This PK activity has been suggested to play a critical role in the transformation activity of this virus. As pointed out above[85] there are two classes of PP, those that dephosphorylate serine/threonine and those that dephosphorylate tyrosine. It would seem that the phosphorylation of tyrosine residues (on certain proteins) is intimately related to the transformation (cancerous) process.[86] If the ability of the body to both recognize that certain critical proteins have been incorrectly phosphorylated at tyrosine residues and remove them is defective, transformation may be facilitated. The regulation of phosphorylation as it affects receptor activity in disease states has great significance in pre- and post-adjunctive therapies which will be discussed more fully.

CHAPTER II — PART III

Cell Regulation Through Epidermal Growth Factor Receptors

Introduction

In the preceding section (Part II) we have discussed cellular phosphorylation processes as they relate to the enzymes which phosphorylate (protein kinase) and dephosphorylate (phosphoprotein phosphatase). In this section there will be described the relationship between protein phosphorylation and the response of the cell to external signals (first messengers) through interactiaon of these messengers with cell surface receptors.

The recipient of live cell injections receives fetal cell receptors of various types which are incorporated into surface membranes of the recipient's cells. The activity of these receptors may be understood in relation to what has been presented previously. The receptor described in this section is that for the epidermal growth factor. (Many other receptors are also regulated by phosphorylation including the insulin receptors to be discussed in Part IV.)

Definition, History and Occurrence

Epidermal growth factor (EGF) is a polypeptide having a molecular weight of 6040.[95] Fragments produced by random proteolytic digestion indicated that the fragment consisting of residues 20-31 was the only active one that enhanced the level of phosphorylation.[96] EGF was first isolated from mouse submaxillary glands in 1962 and was noted to cause precocious eyelid opening and tooth eruption in newborn mice as well as being an epidermal tissue stimulatory factor.[97]

EGF is present in human plasma, urine, colostrum, milk, cerebrospinal fluid and amniotic fluid.[98,99] The normal plasma concentration of EGF in adults is 0.1-0.2 nanograms/liter.[97] The site of EGF production in humans remains to be clarified.[98]

EGF stimulates activity in a wide variety of cells including those of nonepidermal origin and the EGF receptor has a wide tissue distribution. Among the various growth factors isolated, EGF is one of the most potent and best characterized as to its physical, chemical and biological properties.[97] High affinity EGF receptors have been found on human placental cells[95,98,100] as well as the mouse at the tenth day of gestation.[101]

The number of EGF receptors was highest in all fetal tissues at midterm (50% of gestation).[102] This finding is in agreement with those of practitioners of live cell therapy who have found by experience that midterm fetuses have the greatest therapeutic value.

Fetal mouse liver had the highest density of EGF receptors compared with all other tissues analyzed at the 19th day of gestation (90 day term). In the adult the main role of EGF is thought to be the stimulation of tissue repair after damage.[103]

In human placenta the percentages of EGF receptors compared to those of term are during the first trimester, 24.3%, the second trimester, 39.5% and the third trimester, 72%. The number of receptors increased with gestational age.[98]

The total synthesis of EGF has been achieved and consists of 53 amino acids linked together by three disulfide bonds between cysteine residues.[104]

Receptor Structure in Relation to Activity

EGF receptor (EGF-R) is a membrane-bound protein having a molecular weight of approximately 170,000.[117] The part facing

the cytoplasm differs from that external to the cell. That part extending into the cytoplasm is easily detached from that portion within the membrane itself. This part has protein kinase activity, that is, the ability to phosphorylate other proteins. Attached to the part within the cytoplasm is the phosphorylation site, also easily detached. Attached to the external surface are polysaccharide chains which are highly significant in relation to receptor binding of EGF (see Fig. 16).[113,114]

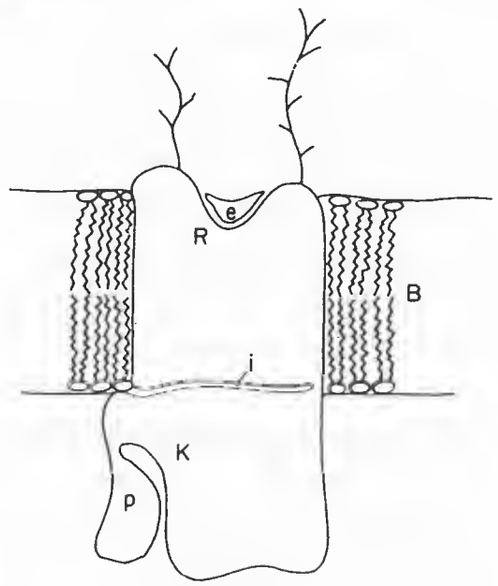


Fig. 16

EGF receptors which have had the addition of polysaccharides (sugar chains) inhibited by an antibiotic (tunicamycin) are incapable of binding EGF. Other experiments indicate there are at least 11 polysaccharide chains linked to the receptor. The presence of these high mannose (a specific sugar similar to glucose) polysaccharides are absolutely necessary for EGF-R to function.[115,116]

The discovery that polysaccharides of a specific type are an absolute necessity for EGF binding and receptor activity may be of great significance in relation to both aging and live cell therapy.

As will be shown below, the biochemistry of mannose is directly related to retinoic acid, a natural derivative of vitamin A. Defective polysaccharide structure resulting from aging or disease will lead to impaired EGF binding. We shall see below that as a result of fetal cell injections (live cell therapy) high affinity EGF receptors are incorporated into the patient's outer cell membranes, replacing defective receptors which may be present.

Response to EGF Binding

EGF is one of the best studied of a growing class of polypeptide cell regulators that not only stimulate cell proliferation but modulate a variety of other cell functions.[105] Events that occur shortly after binding EGF (within minutes) include an increase in phosphotidylinositol turnover (discussed above),[72] enhanced phosphorylation of cell proteins,[106] increased nutrient uptake including amino acids and glucose[107] and increased ion flux.[108] Delayed effects of EGF binding (hours to tens of hours) include the stimulation of RNA and DNA synthesis,[107] an elevation of 2',5'-oligoadenylate synthetase (an enzyme that synthesizes short lengths of poly-A RNA)[109] and an increase in the activation of a variety of other enzyme systems.[110,111]

EGF Receptors in Relation to Aging

Senescent human uterine smooth muscle cells have abundant receptors but respond very poorly to EGF.[112] This poor response may result from defective bound polysaccharides on the receptor surface or an internal defect.

Studies with a particular strain of cultured embryonic lung fibroblasts showed that the protein kinase activity of EGF-R (ability to phosphorylate protein) was markedly reduced in old receptors.[118]

This finding is again related to live cell therapy in replacing defective, aged receptors with functional ones.

In cultured embryonic epidermis of the mouse, EGF receptors found on epithelial cells have the ability to cause the proliferation of these cells (when stimulated by EGF). In contrast, skin tissues which have started to differentiate lose their growth potential and carry either an undetectable or sharply reduced number of EGF-R which are important in the control of epithelial proliferation.[119] These findings are highly significant when considering that the skin is constantly replenishing itself from beneath and that the older, dead cells are on the surface. This replenishment process is also true of the inner lining of the intestine (epithelial cells) which is constantly being replenished through the action of EGF and its receptors.[97] A deficiency in the replenishment of intestinal epithelial cells results in impaired food absorption and improper nutrition.

Affinity of Receptors for EGF

There is much evidence indicating that the EGF receptor may be found in either of two states in relation to its ability to bind and respond to EGF. Thus, there are found high affinity receptors and low affinity receptors. In some experiments it was shown that even though there was an inhibition of EGF binding, the total number of receptors remained constant.[120] Those factors which are known to cause the activation of protein kinase C also result in the production of low affinity EGF receptors.

It is implied by these results that excessive phosphorylation may lead to an undesirable situation unless the dephosphorylation process is fully functional to maintain the proper balance between these two opposing activities. This mechanism may represent a form of regulation to the activity of EGF since the increased

phosphorylation occurs only in the presence of EGF.[121]

In a study of EGF receptors in embryonic mice it was found that the number of receptors increases during gestation for all tissues examined. However, the affinity of the receptors declines for carcass and placenta but remains relatively unchanged for brain and liver. These observations suggest that EGF may initially stimulate proliferation in embryonic cells and then stimulate differentiation. More will be said about this aspect below in this section, Significance of the Inner and Outer Placenta. In the adult its main role may be to stimulate tissue repair.[103]

In studies of human fibroblasts the presence of the nonsteroid antiinflammatory drug, dexamethasone, stimulated the response to EGF 4 to 5 times and was accompanied by the appearance of a population of high affinity receptors not detected in the untreated cells.[122] Dexamethasone is known to inhibit the synthesis of certain prostaglandins which may suggest an association between prostaglandins and the regulation of phosphorylation/dephosphorylation.

Treatment of a cell with neuraminidase (sialidase) increases the ability to bind EGF.[122] Sialidase is an enzyme that removes terminal sialic acid from polysaccharide chains. The sugar immediately adjacent to sialic acid is always galactose. Thus, treatment of fetal cells with sialidase will increase the ability to bind EGF.

The ability of the EGF receptor to phosphorylate proteins (protein kinase activity) requires the presence of either manganese or magnesium ions but not calcium. In these experiments EGF-enhanced phosphorylation was detectable within 15 seconds and was clearly evident at 30 seconds.[123]

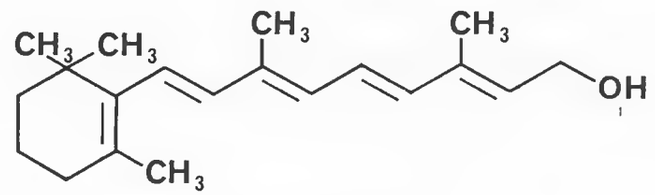
As mentioned above, the EGF receptor has the ability to phosphorylate other membrane bound proteins including other EGF receptors (autophosphorylation). The amino acid residue phosphorylated is tyrosine which makes this protein kinase activity different

from that of the cytoplasmic protein kinases which phosphorylate serine and threonine residues. This difference will be discussed in greater detail below under the heading, Relationship of EGF and EGF-R to Cancer.

The autophosphorylation of EGF receptors is stimulated by dimethylsulfoxide (DMSO). It is thought that kinase activity is stimulated by means of a conformational change in the EGF receptor.[124] (See Fig. 16.)

The Significance of Retinoic Acid in EGF Receptor Formation

The structure of retinol (vitamin A) shown in Fig. 17 is seen to consist in part of a long chain unsaturated hydrocarbon ending in an alcohol group (-OH). When this group is oxidized by normal metabolic processes a carboxylic acid (-COOH) results forming retinoic acid (RA). Thus, RA is a normal metabolite of retinol.[146]



Retinol
Fig. 17

Carotenoids are probably less active biologically than RA because they are less efficiently absorbed from the intestinal tract.[146]

Intraperitoneal injection of RA is more effective than oral administration because of poor absorption through the gut. Within a few hours after feeding RA to experimental animals no RA is detected nor is there a storage for RA in the body. For these reasons RA is not commercially available as a dietary supplement.[146]

RA is transported into the general circulation in the form of the carboxylic acid anion, bound to serum albumin. Retinol-binding protein (RBP) is found in the cytoplasm and is thought to participate in the biochemical activities of retinol.[146]

There are many examples in biochemistry of polysaccharides consisting of chains of the sugar mannose. Investigations into the mechanism of formation indicates the participation of vitamin A and,in particular, its metabolic product, RA. The incorporation of mannose into glycopeptides was greatly reduced in vitamin A deficiency. However, the administration of RA was as effective in restoring this incorporation as vitamin A.[146]

The incorporation of galactose was also affected by the deficiency of retinoic acid (RA). Many polysaccharide chains are terminated by sialic acid which is always bound to galactose. Without the terminal sialic acid many glycoproteins are non-functional and are removed from the circulation by the liver. These considerations point to the importance of RA in the completion of glycoprotein biosynthesis.[146,149]

When retinol (also known as vitamin A alcohol) is oxidized, RA is the result. Both retinol and RA function as carriers of sugar in their phosphorylated form in which the terminal OH group reacts with a hydrogen of the phosphate group thereby forming water and the phosphorylated derivative.

As mentioned briefly above, the EGF receptor carries on its outer surface many mannose-rich polysaccharides, without which

the receptor cannot function. Retinoic acid enhances the binding of EGF to its receptor, not by increasing the affinity of one for the other, but by increasing the number of receptors on the cell surface.[147] A comparison made for EGF binding enhancement in 3T3 cells and the virally transformed cell, 3T3SV (simian virus), revealed that RA enhanced EGF binding in normal 3T3 cells but not in the transformed 3T3SV cells. This difference might be explained by the presence of the retinoic acid binding protein in normal 3T3 cells and its absence in transformed 3T3SV cells.

These combined results are in agreement with the finding that EGF receptors are deficient in transformed cells. The absence of retinoic acid binding protein from transformed 3T3 cells inhibits the formation of a functional EGF receptor resulting from the inability to attach high mannose polysaccharides to the receptor protein. This results in a deficiency of functional EGF receptors on the cell surface which, in turn, leads to defective phosphorylation of vital cellular proteins necessary for normal cell activity.[148]

These results with both experimental animals and cultured cells indicate the great significance in live cell therapy of adequate vitamin A (and its metabolic product, RA). The administration of live cells provides EGF receptors to the surface of the patient's own cells. The presence of fully functional receptors on these cells enables EGF to generate important cytoplasmic signals (phosphorylation) to the cell, activating long dormant processes and restoring cell vitality. As a result of this long overdue stimulation the cell once again has the capability of synthesizing its own EGF (and other) receptors so vital to normal metabolism. This end result may be accomplished only if sufficient vitamin A is available for the complete biosynthesis of this and other cell surface receptors.

It has also been shown that retinoic acid induces the differentiation of certain embryonal carcinoma cells (PCC4aza 1R). After

1 to 2 days treatment with RA these cells differentiate irreversibly into a cell type with fibroblastic morphology. The original cells show very low levels of EGF binding whereas the differentiated cell shows much higher levels of binding. Similar results have recently been reported for other embryonal carcinoma cell lines.[147]

Retinoic acid has also been shown to inhibit the biosynthesis of cholesterol.[150]

Experimental rats maintained on retinol-deficient diets show testicular atrophy and loss of the terminal epithelium. Serum testosterone levels in these rats were lower than in controls. However, retinoic acid-fed rats had serum testosterone concentrations similar to those of normal rats.[151]

EGF Binding Inhibited by Reactive Oxygen Toxic Species (ROTS)

Vitamin K_3 (menadione) which is chemically, 2-methyl-1,4-naphthoquinone, markedly inhibits the binding of EGF to its receptor. The same molecule without the methyl group (1,4-naphthoquinone) is almost as potent an inhibitor as the vitamin. It has been shown that if the benzene ring is also removed (yielding 1,4-benzoquinone) the inhibitory activity is still seen. These chemical modifications suggest that the essential requirement for inhibition is simply the para-quinone structure (more inhibitory than an ortho-quinone). (See Fig. 18.)

Vitamin K_3 treatment (or any simple p-quinone) seems to modulate EGF binding by decreasing the affinity of the receptors for EGF. It was also indicated that vitamin K_3 is probably metabolized in the cell to compound(s) that cause the inhibition.[125] As indicated above, the loss of EGF receptor affinity results from phosphorylation by activated protein kinase C.[120]

Vitamin K_3 is known to generate hydrogen peroxide through normal metabolic processes.[126] It is also known that the reduction

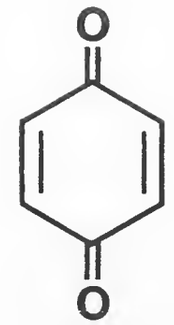


Fig. 18

of a quinone leads to a bisphenol which combines with another quinone molecule yielding a semiquinone (a free radical).[127] Thus, from two standpoints the inhibition of EGF binding to its receptor implicates hydrogen peroxide and/or free radicals as a mechanism, both of which are members of the ROTS family of toxic oxygen metabolites.

Still another implication of ROTS in EGF binding is that the receptors for both EGF and insulin are sensitive to digestion by trypsin (a proteolytic enzyme). At concentrations as low as 50-200 micrograms the EGF receptor is destroyed by trypsin.[128] Trypsin is normally inhibited by the alpha$_1$-antitrypsin inhibitor which has four methionine residues in the active site. The amino acid, methionine, having the grouping, $-CH_2SCH_3$ as a residue, is subject to oxidation by hydroxyl radical, converting methionine to methionine sulfoxide, $-CH_2S(O)_2CH_3$, resulting in total inactivation.[129]

In all degenerative diseases studied the level of ROTS is increased over normal values.[87] It is conceivable that the generation of hydroxyl radical through the combination of hydrogen peroxide and superoxide (Haber-Weiss reaction) results in the inactivation

of the trypsin inhibitor followed by the destruction of the EGF receptor through the action of trypsin. These considerations may explain why those having a variety of degenerative diseases benefit from live cell therapy which replaces nonfunctional EGF and other cell surface receptors with those which are fully operative. The use of pre-therapy to minimize systemic effects of various pathological conditions and the use of antioxidants is an important consideration in live cell therapy.

Degradation of EGF Receptors by Internalization

The binding of EGF to dispersed receptors on the cell surface is followed by receptor aggregation and internalization into pinocytic vesicles or "receptosomes" which are small subcellular sacs resembling lysosomes (see Fig. 19). The receptosomes may then follow either of two pathways. (1) The first pathway is the merging of the receptosome with vesicles apparently originating with the Golgi apparatus. This is a nondegradative pathway with the receptors being recycled to the membrane. (2) The second pathway results in rapid degradation of the receptor with probable merging with lysomes.[130] (See Figs. 20 and 21.)

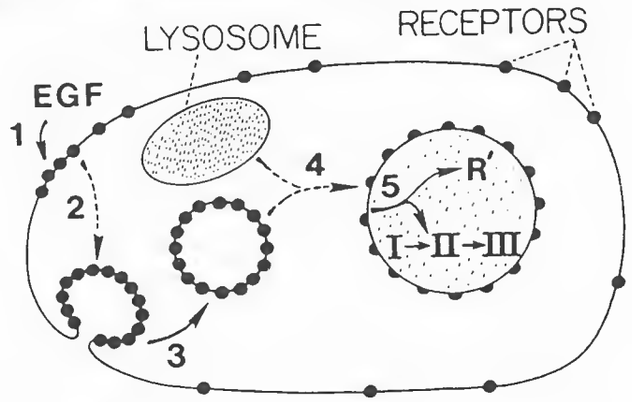


Fig. 19

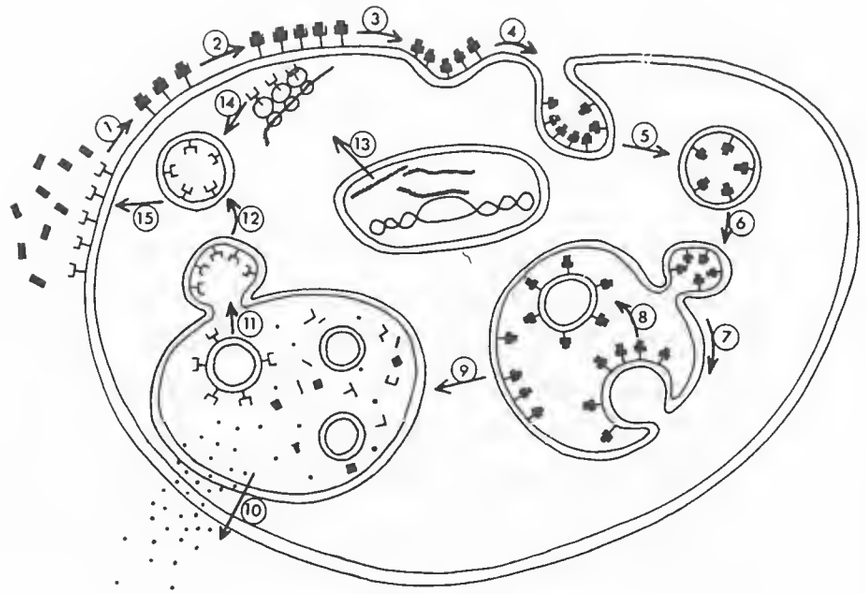


Fig. 20

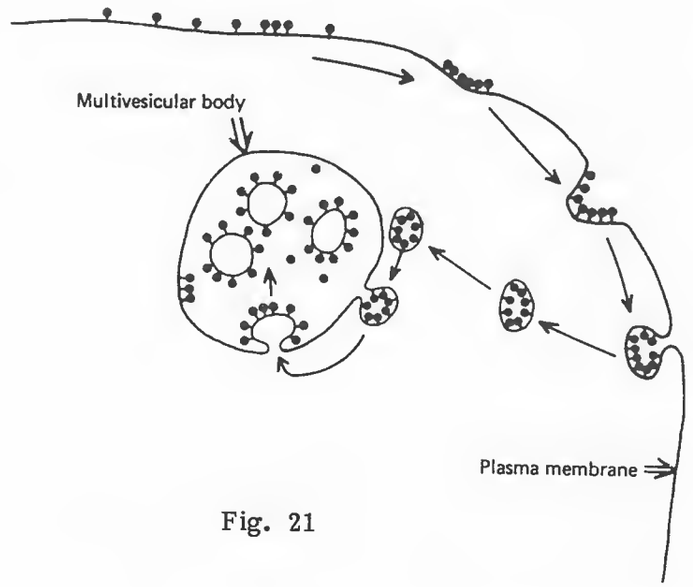


Fig. 21

The extent to which the receptors are processed in the two pathways is determined by the physiological state of the cell. Uptake into the Golgi vesicles occurs under conditions of adequate nutrition. Incorporation into lysosomes and degradation occur under conditions of amino acid deprivation.

These observations have implications in live cell therapy. When receiving receptors from fetal cells (by injection) it would be desirable to have the receptors take the nondegradative pathway for as long as possible. This implies good nutrition (free amino acid supplementation if necessary prior to and immediately following live cell injections).

The other degradative pathway may explain one of the values of fasting in which defective receptors would be internalized and destroyed, subsequently replaced by newly synthesized ones, hopefully of better quality.

In either pathway the degradation of EGF receptors does not mean that there would be little or no value from live cell injections. The presence of functional EGF receptors on the surface of a cell for only a brief period is sufficient to elicit a response from external stimuli which may reach to the nucleus of the cell and have far-reaching effects.

In one cell type (3T3) about 80% of the endocytosed receptors were recycled back to the plasma membrane under certain conditions[97] but under other conditions approximately 98% were recycled to the surface.[131] The binding of EGF to the receptor need not lead to the proteolytic processing of the receptor.[105]

Down Regulation of EGF Receptors

As a means of regulating the response of the cell to EGF, a phenomena known as "down regulation" is observed. Subsaturating amounts of EGF cause a decreased ability to bind after exposure

to the cell. It has been shown to be caused by the loss of cell surface receptors and may act as a natural controlling mechanism since the extent of internalization is dependent on the concentration of EGF.[122]

The internalization of EGF receptors is preceded by a process known as "clustering" in which dispersed receptors aggregate and apparently are covalently linked together. The entire clustered area is pinocytosed and internalized as described above. It is believed that the rapidly stimulated responses to EGF (tens of minutes to hours) result from microclustering within the plasma membrane while those that require a long period of cell stimulation (for example, cell division, requiring tens of hours) result from receptor internalization.[105]

EGF Cannot Cause Cancer

Those who are given fetal cell injections will have EGF receptors incorporated into the plasma membrane of many types of their own cells. Some may be fearful that since EGF is capable of stimulating DNA replication and cell division, cancer may result from increasing the performance of these processes. However, it is well known that cell division, such as is seen in wound healing or replacement of dead cells does not represent the cancerous state. One difference between the cancerous state and the normal process of cell division and growth is that in relation to cancer, the mechanism by which the cells recognize that growth should cease is defective or non-existent. For a cell to be in this state it (or its ancestors) must have been "transformed." The causes of cell transformation are many and may include the carcinogens, chemical substances having specific activities in relation to cell

function. Also included among cancer causing agents are certain viruses which are only chemicals (viruses are not microorganisms nor "alive").

A simple single-signal process involving receptor-EGF interaction with the production of a single second messenger cannot explain the action of EGF. It seems likely that multiple signals are necessary and these are generated by the EGF-receptor complex at various times. Clustering may generate one of these signals while another may be generated as a result of protein phosphorylation but none of these alone is sufficient for entry into the DNA synthetic phase.[97] (See Fig. 20.)

Transfer of Receptors From Membranes to Cells

A discovery having great significance explaining in part the biochemical mechanism of action of live cell therapy shows that cell membrane receptors may be transferred from membrane fragments into the outer membranes of intact, living cells. The plasma or outer membranes from mouse liver cells were isolated and incubated with a particular type of cell having no EGF receptors. The varient cell line, NR-6, derived from mouse 3T3 cells (fibroblasts), cannot respond to EGF because of this deficiency.

Experiments were performed to determine if membrane receptors from another cell could be incorporated into the membranes of NR-6 cells by a natural physiological mechanism (without added substances to promote membrane fusion). This cell type was chosen for these experiments because, if EGF receptors were incorporated into the membranes of NR-6 cells, tests could be made to determine if there was a response to added EGF which, without EGF receptors, is normally not seen.[132]

Results from such experiments showed that receptors were, in fact, incorporated by a natural process into NR-6 cell membranes. The inserted receptors could bind radiolabeled (radioactive) EGF (^{125}I-EGF) with high specificity and affinity relative to other proteins also present in the added membranes. The number of EGF receptors that could be inserted increased in proportion to the quantity of donor membranes. At maximal insertion approximately one million receptors were incorporated per NR-6 cell. The parent 3T3 cell line from which the NR-6 cell was derived normally contains only 100,000 receptors per cell.[132]

The inserted receptor was exceptionally stable to dissociation and other losses. The addition of EGF stimulated DNA synthesis in a concentration-dependent manner. In contrast, NR-6 cells not treated with membranes were totally unresponsive to EGF.

In normal cells response to EGF results in the activation of the enzyme, phosphofructokinase. In untreated NR-6 cells this enzyme is not activated by added EGF. However, in NR-6 cells treated with liver cell membranes (carrying incorporated EGF receptors) a marked response to added EGF was seen resulting in a twofold activation of phosphofructokinase.

In normal 3T3 cells EGF also stimulates the uptake of alpha-aminoisobutyrate. A 20% stimulation of this amino acid was seen when cells containing inserted receptors were incubated with EGF. Although low, this stimulation is comparable to that obtained with normal receptor-containing cells.[133]

As indicated above, these experiments provide proof for a part of the biochemical mechanism of action of Live Cell Therapy. It has been stated above that fetal cell membranes carry EGF (and other) receptors. Following injection, membranes resulting from the breakdown of these cells merge with the plasma membranes of the recipient's cells and incorporate EGF receptors. The contact

of endogenous EGF results in the cell responses enumerated above; namely, stimulation of amino acid uptake, stimulation of DNA replication, cell repair, enzyme activation, etc. These combined cellular processes and others unknown will most certainly have a Rejuvenative Effect on cell performance.

Significance of the Inner and Outer Placenta in Live Cell Therapy

The whole placenta consists of the inner placenta and the outer placenta. The inner placenta has three times the number of EGF receptors as the outer placenta but of lower affinity for EGF. In a study of various mouse embryonic tissues from day 12 to the adult the types of EGF receptors present and their numbers was determined. It was found that in the early stages of development there were present receptors having high affinity but fewer numbers than those found later. As the number of receptors increased the affinity for EGF decreased. At the adult stage there are present many more receptors than in the embryo but having greatly reduced affinity for EGF.

These results associate a state of rapid proliferation of relatively undifferentiated cells (early embryonic) with few receptors having a high affinity for EGF. There is also found an association between a state of differentiation and the presence of many more receptors having low affinity for EGF.[103] As described above (Part II), protein kinase type I was found in high levels during embryogenesis but decreased during later stages of development. Type II protein kinase, on the other hand, was found to decrease with differentiation (see Fig. 22).[66]

INNER PLACENTA	OUTER PLACENTA
DIFFERENTIATION	NONDIFFERENTIATION (Proliferation)
MANY RECEPTORS	FEW RECEPTORS
LOW AFFINITY	HIGH AFFINITY
TYPE II PROTEIN KINASE	TYPE I PROTEIN KINASE
FOR CANCER THERAPY	FOR HEART THERAPY

Fig. 22

It is the ratio between protein kinase I and II that apparently determines the state of cellular activity; that is, whether a cell is relatively nondifferentiated and highly proliferative or differentiated with little proliferation.[130] The balance between the two types of protein kinase is in turn, at least partly determined by the affinity of the EGF receptor for EGF.

In treating cancer it is desirable to cause the cells to become more differentiated since cancerous cells are in a less differentiated state than normal. It is also desirable not to stimulate proliferation since cancer cells are already in a state of uncontrollable replication. Both of these conditions may be met by giving only the inner placenta to cancer patients.

In contrast, as described above, there was found in experimental cardiac hypertrophy an increase in protein kinase type I.[105] In this condition the proliferation of cells is desirable, to replace those damaged by the experimental drug used. When this drug was withdrawn the level of protein kinase type I returned to normal. These results combined with those given above indicate that the outer placenta would be the tissue of choice in treating a patient with heart damage (see Fig. 22).

Significance of Liver Live Cells in Cancer Treatment

Liver binds the greatest amount of EGF because of the high density of EGF receptors. However, these are low affinity receptors associated with type II PK related to cellular differentiation. Cancer cells, being in a dedifferentiated state (transformed) would become more differentiated by any agent causing differentiation. For these reasons live cell liver is one of the tissues of choice in treating cancer.

Significance of EGF to the Sodium/Hydrogen Exchanger

Cellular pH is regulated through the activation of the sodium/hydrogen (Na^+/H^+) exchanger. This is an energy-requiring pump (membrane bound) which transports acid (H^+) out of a cell while simultaneously transporting sodium (Na^+) in. This equal exchange of positive charges (ions) is necessary to maintain electrical neutrality. Hydrogen ion (H^+) is the cause of acidity, the greater the concentration, the lower the pH. On the other hand, the presence of sodium ions (Na^+) raises the pH resulting in less acidity (more basic). If the pH of a cell is low (acidic) and H^+

is pumped out and simultaneously, sodium ion is pumped in, the pH of the cell will rise.

Evidence that cytoplasmic pH may play an important role in the metabolic activation of quiescent cells (inactive or latent) has recently been discovered.[72] In particular, the stimulation of growth in mammalian fibroblasts leads to a rapid increase in cellular pH; in fact, this alkalinization is necessary for the initiation of DNA synthesis.[135]

It has been shown that the application of EGF activates the Na^+/H^+ exchanger causing a rise in cellular pH.[136] The mechanism of this response has been shown to be that described above, beginning with the breakdown of inositol phospholipids resulting in an increase of diacylglycerols which, in turn, stimulate the activity of PK-C. Activated protein kinase C catalyzes the phosphorylation of serine and threonine residues on the EGF receptor resulting in a decrease in the tyrosine-specific protein kinase activity of this receptor.[137,138] More will be said of this phosphorylation process below.

The Na^+/H^+ exchanger in cancer cells is known to be defective. For this reason and others the pH of cancer cells is usually lower than normal cells. It would, therefore, be advantageous to activate the Na^+/H^+ exchange mechanism in cancer cells in an effort to restore cellular pH values to normal. For these reasons the incorporation of EGF receptors into the plasma membrane of cancer cells (by live cell therapy) and the activation of these receptors by EGF is highly significant as a technique in the treatment of cancer.

The little known dietary mineral, vanadium (vanadate ion), activates the Na^+/H^+ exchanger by a mechanism similar to that of EGF or other polypeptide growth factors. Thus, vanadium may be considered an effective adjunctive agent to live cells in the

treatment of cancer, assisting in the restoration of the acid-base balance.[139]

A dietary source of vanadium is black pepper, possibly black sesame seeds as well as the Oriental black mushroom, highly prized for its putative longevity property. These foods may be rich sources of vanadium because each contains black melanin, a polymeric substance similar to some modern plastics. The melanins (known to be present in mushrooms) carry charged groups which act as chelators of odd metals. This has been verified through mineral analyses of various melanins. Another natural melanin is found in the ink of the cuttlefish or squid, known as sepia.

Relationship of EGF and its Receptor to Cancer

As indicated above, cancer cells are said to have undergone transformation, a cellular process resulting in contact inhibition (loss of ability to bind together) and uncontrolled replication. Studies to determine some of the essentials of this process at the biochemical level have shown that there is a continuous decrease in the number of EGF receptors on the cell surface.[140]

In a similar study Chinese hamster embryo cells lost cell surface EGF receptors gradually during the transformation process until only 10% of the original number were present.[141]

Both murine (rodent family) and feline (cat family) RNA tumor viruses (sarcoma type) are capable of causing cell transformation. Associated with transformation is a rapid and profound loss of detectable membrane receptors for EGF. This is not seen in cells transformed by the DNA viruses, SV40 or polyoma types.[145]

This finding may have significance in relation to the RNA virus thought to be the infectious agent in AIDS (HTLV-III). The normal flow of genetic information in a cell is from DNA to RNA

to protein. An RNA virus (consisting of both RNA and a protein coat) must replicate both its RNA and protein. The viral RNA is fully capable of replicating the protein coat but the replication of viral RNA presents a problem to the virus since RNA cannot replicate itself. This problem is solved, however, by a segment of viral RNA that codes for an enzyme (protein) known as reverse transcriptase which generates a segment of DNA corresponding to the viral RNA. The "mirror image" strand of DNA is fully capable of replicating any number of viral RNA strands identical to the original RNA virus. Thus, an RNA virus replicates in two parts, protein and nucleic acid (RNA).

Since EGF receptors are present on certain white cells (the cellular aspect of the immune system), one action of the AIDS virus may be to inhibit the binding of EGF to these receptors thereby impairing normal immunologic activity.

Rous sarcoma virus caused transformed cells to lose one class of high affinity EGF receptors while a class of low affinity receptors not only did not down regulate but did not respond to high concentrations of EGF.[122]

Most transforming DNA viruses do not influence the number of EGF receptors but one exception has been reported. Kidney fibroblasts transformed by SV40 virus showed up to 90% reduction of receptors for EGF. The remaining receptors bound EGF with about three times the affinity than normal receptors. However, the receptor in the transformed cell differed in its glycosylation.[142]

Transformation of mouse embryonic cells by the carcinogen, 3-methylcholanthrene, resulted in the complete loss of EGF receptors.[122]

A-431 cells (a human epithelioid carcinoma) have more than two million EGF receptors per cell yet are totally unresponsive to EGF whereas another cell line (OC15, an embryonal carcinoma)

responds favorably with only 30,000 receptors per cell.[145] However, when exposed to EGF, membranes isolated from A-431 cells result in the phosphorylation of endogenous membrane proteins including the EGF receptor. As mentioned above, the phosphorylation is specific for tyrosine residues rather than those of serine and threonine, characteristic of protein kinases in general.[97]

It is interesting to note that the Rous sarcoma virus has coded within its genetic material a protein kinase specific for tyrosine residues.[134,144] The activity of the enzyme is correlated with the appearance of transformation.[146]

As indicated above, the protein kinase (phosphorylating) activity of the EGF receptor is tyrosine-specific. This fact, coupled with the tyrosine-specific activity of the kinases derived from RNA viruses, implies that in the process of transformation the virus is attempting to mimic (and possibly exaggerate) a natural process intended to stimulate normal cell growth. Thus, it may be said that, at the molecular level, at least one contributing factor in the cause of cancer is the phosphorylation of the specific amino acid, tyrosine, rather than those of serine and threonine. From the wealth of information presented above in relation to the significance of protein phosphorylation it may be appreciated that such a simple modification of this process may have far-reaching consequences in relation to live cell therapy.

CHAPTER II — PART IV

The Insulin Receptor and its Relation to Live Cell Therapy

Introduction

In Part II, Phosphorylation and Cell Surface Receptor Activity, and Part III, the relationship between a hormone or growth factor and its receptor was described, resulting in a cellular response. Everyone is familiar with the hormone, insulin, but few may realize that the action of insulin is through a specific cell surface receptor which, upon contact with insulin, changes its configuration (shape) stimulating a variety of metabolic processes.

It will be shown below that certain metabolic diseases of sugar metabolism are characterized by either defective or deficient insulin receptors. Also of interest is the realization that middle-aged persons have only half the insulin receptors of younger people.

From these and other pertinent facts brought out below, it will become apparent how receiving insulin receptors through the administration of appropriate live cells benefits those who are in need for greater cellular response from insulin.

The Insulin Receptor — Physical Properties and Structure

The insulin receptor is a membrane-bound glycoprotein (consisting of both protein and carbohydrate) formed of four units bound together by disulfide bonds. Two of the subunits are identical (designated "alpha") with the remining two (designated "beta") also identical.

The two alpha subunits have an apparent molecular weight of 120,000-130,000 while the two beta subunits have an apparent molecular weight of 90,000. It is thought that portions of both

subunits are exposed on the cell surface and that the beta subunit spans the membrane bilayer.[157]

Following binding of insulin by the receptor the resulting complex activates an "effector system" which begins a cascade of events leading to the effects characteristic of insulin such as protein synthesis, stimulation of both amino acid and glucose transport, enzyme activation or inactivation and others.[152]

Following synthesis the polypeptides destined to become the alpha and beta subunits of the insulin receptor receive polysaccharide chains (sugars) bound to specific amino acids (glycosylation). The significance of these polysaccharides is seen when an intact receptor is treated with neuraminidase and beta-galactosidase, two enzymes which remove sialic acid and galactose, respectively. These two sugars (in this sequence), known as "capping sugars," are found on the terminal ends of many polysaccharides. Without their presence on the insulin receptor the capacity to bind insulin is markedly decreased.[156]

Targets for Insulin Action

The most important cell types upon which insulin acts to regulate the balance of glucose are muscle cells, hepatocytes (liver cells) and adipocytes (fat-storing cells). Of the circulating cells both erythrocytes (red blood cells) and monocytes (a class of white blood cell) are the most studied.[155] Insulin receptors have also been found on cells not ordinarily considered as targets for insulin action; namely, both B- and T-lymphocytes.[154] More will be said below of the significance of this discovery.

Insulin Receptors on Fetal Cells

The most significant single fact in relation to live cell therapy and the study of insulin action is that insulin receptors are found in great abundance on fetal cells; in fact, in greater numbers than in adult tissues. This, at first glance, seems unjustified but may be understood by realizing that the action of insulin is related to growth processes. For a cell to divide it must be in an active state of protein synthesis which requires an abundant supply of amino acids. These basic building blocks of protein fall naturally into three groups, the basic types (arginine, lysine), the acidic types (aspartic and glutamic acid) and the neutral types (glycine, alanine, valine, for example). Each of these classes of amino acids is transported across the plasma membrane into the cell by a process requiring energy. This "active transport" of amino acids into cells is stimulated through insulin action.

Secondly, the metabolism of glucose is recognized as an energy source and is certainly an important requirement in a developing organism.

As we shall see below, several well-recognized human diseases as well as aging have been shown to involve reduced numbers of insulin receptors. This deficiency may be compensated through the administration of live cells carrying the very receptors which are so urgently needed.

Fetal muscle cells develop an increasing density of insulin receptors during embryonic life.[162] Insulin binding by human liver membranes has been studied in fetuses. Receptor number increased from 15 to 25 weeks but not thereafter. This discovery confirms the practice of using in live cell therapy an animal fetus not much older than 50% of term.

A study of insulin binding by fetal lung tissues showed that these fetal membranes had a fivefold increase in binding capacity of high-affinity receptors as compared to adult tissues.[168]

Insulin receptors have been demonstrated on embryonic brain cortical cells. These receptors showed high affinity insulin binding and appeared to be different from those of more differentiated (adult) cells in their increased binding ability.[165,173]

Other studies have shown that insulin receptors are particularly abundant in discrete portions of the brain, such as the hypothalamus.[160]

These studies in relation to brain tissues are of great significance in the practice of live cell therapy. The implications are that diseases which are not diabetes-related (the disease most often thought of in relation to insulin) may be treated with insulin receptors found in neurological and other tissues. Conversely, diabetes and related sugar dysfunction diseases may be treated with brain and other tissues rich in insulin receptors. These seemingly unrelated associations between certain tissues used successfully in the treatment of specific diseases, long known by practitioners of live cell therapy, are confirmed through the establishment of the presence of insulin receptors on those tissues.

Research conducted with human placentas ranging in age from 26 weeks to term (36 weeks) showed that maximum binding of insulin occurred before the 26th week. This again confirms the practice of selecting a fetus not much greater than 50% of term.[167]

Specific insulin binding sites have been demonstrated in placental membranes[164,169] in addition to unique receptors for the insulin-like peptides[164] about which more will be said below.

Heart muscle contains receptors for both insulin and the insulin-like peptides.[163] Insulin receptors are also present in kidney tubules and glomeruli.[160] The fetus has high concentrations of

insulin receptors in many tissues which begin to appear early and may outnumber those found in adult tissues.[160]

Phosphorylation and Dephosphorylation of the Insulin Receptor

The cascade of biochemical events immediately following insulin binding to its receptor is not fully understood. However, it is known that one of the earliest effects is the phosphorylation of the receptor in the beta-subunit.[154] The amino acids phosphorylated are serine and tyrosine.[157]

Evidence indicates that there may be another protein kinase responsible for the receptor phosphorylation.[159] Regardless of the causative agent it has been demonstrated that the insulin-dependent phosphorylation of the receptor enables it to phosphorylate other proteins on tyrosine residues.[159] A reasonable hypothesis which has been advanced is that phosphorylation of tyrosine residues on select target proteins is responsible for some if not all of the cellular actions of insulin.[159]

The phosphorylated receptor remains active as a protein kinase following the removal of insulin which indicates that dephosphorylation of the receptor rather than dissociation of insulin terminated the insulin signal.[159] It has also been shown that the dephosphorylation of the receptor (termination of insulin action) results from another agent (the receptor is not auto-dephosphorylated).[174]

The Second Messenger for Insulin Action

Many hormones and related cellular factors have their actions mediated by cAMP (cyclic AMP) as described elsewhere in this

text. For this reason cAMP is known as the "second messenger" for hormonal action.

There is a different story with insulin, however, experiments indicating that the opposite response of cAMP may be occurring. Inhibition of cAMP may occur after insulin binding resulting from an inhibition of the enzyme responsible for its formation (adenyl cyclase) and an activation of an enzyme which destroys it (cAMP phosphodiesterase).[160]

Despite extensive efforts the second messenger for insulin has escaped positive identification.[160] However, a short length peptide having from 10-15 amino acid residues has been isolated from muscle tissue which inhibits cAMP-dependent protein kinase (a phosphorylating enzyme) and also activates phosphoprotein phosphatase (a dephosphorylating enzyme). The peptide also mimics insulin in activating mitochondrial pyruvate dehydrogenase.[166]

Regulation of Insulin Response

The response to insulin is regulated by several different mechanisms, each described separately below.

Modification of Receptor Affinity

Contrary to what might be expected, the affinity of insulin receptors progressively decreases as more receptor sites are occupied. At low physiologic concentrations of insulin most receptor sites are empty with the receptors being in their highest state of affinity. With increasing receptor occupancy by insulin the affinity of the remaining receptors decreases.[152] For maximum activation of glucose transport, cells require only from 5-10% occupancy.[155,156] The biochemical mechanism for some of the changes in receptor affinity may be related to the association or clustering

of receptors within the membrane possibly involving the formation of covalent bonds between them. This type of response to the presence of insulin thus constitutes a means of preventing an overreaction to excessive amounts of insulin.

The binding of insulin to its receptor is highly sensitive to changes in pH. A pH of 6.8 (normal plasma is 7.4) can reduce binding by 50%.[158]

Certain divalent cations (minerals having a charge of +2) have been shown to increase insulin binding in a variety of tissues. Both calcium and magnesium ions increase the binding of insulin to its receptor.[158]

Recycling of Receptors (Down-Regulation)

As indicated above, the response of a cell to insulin begins at the membrane as a response to the binding of insulin to a specific membrane-bound receptor. As the number of receptors on a cell decreases the response to a given amount of insulin also decreases. Thus, a second mechanism by which the cell may regulate the response to insulin is to remove receptors from the surface thereby lessening the number of occupied sites. This process is known as internalization or down-regulation. When insulin binds to its receptors there follows an aggregation of these receptors into pitted regions of the plasma membrane. Aggregates of many receptors are then taken into the cell by endocytosis (the pinching off of a small bubble or membrane) where they fuse with the Golgi structure (a permanent membraneous subcellular body). Here it is thought that insulin is separated from the receptor and eventually enters lysosomes for digestion and destruction.[155,156] In most cells the major fraction of internalized receptors is recycled to the cell membrane.[161] (See Fig. 23.)

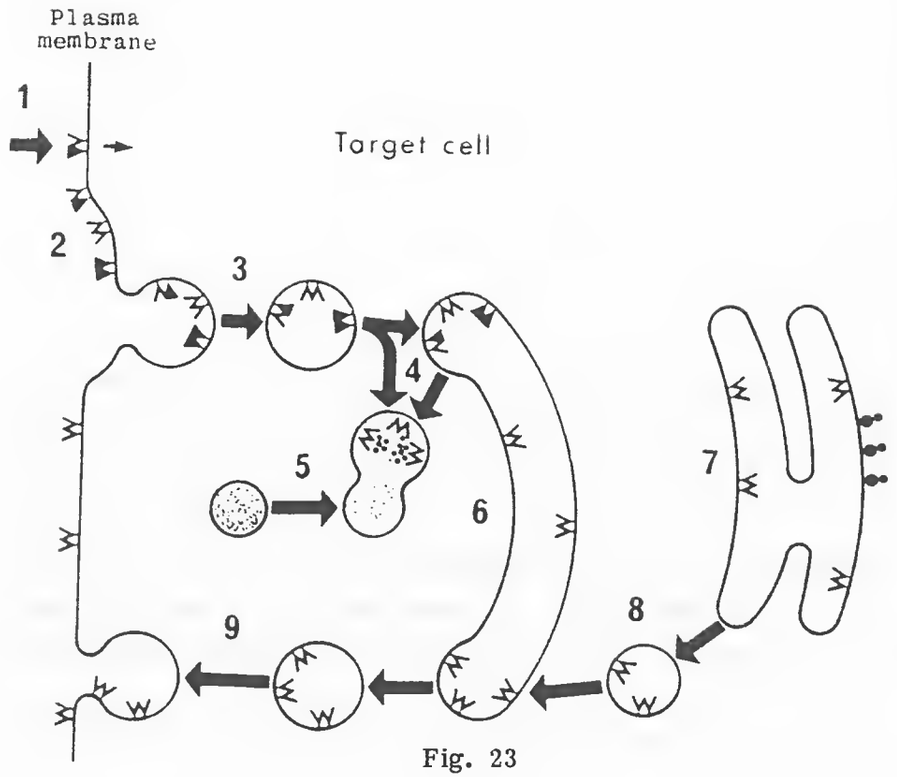


Fig. 23

The binding of insulin to plasma membrane receptors stimulates the production of new receptors.[155] Experiments indicate that the continued synthesis of a short-lived protein is essential for down-regulation as well as the degradation of the receptor.[156]

A number of agents capable of eliciting the biological responses induced by insulin have the capacity to cause down-regulation of the insulin receptor. These agents include antibodies to the insulin receptor, vitamin K_5, spermine and hydrogen peroxide. These substances mimic insulin action and cause a decrease in cell surface insulin receptor levels. These agents may induce a common configurational change in the insulin receptor resulting in "insulin action" and down-regulation.[156]

In many if not all degenerative diseases there is an increase in the serum level of hydrogen peroxide.[87] In patients with these diseases the increased level of hydrogen peroxide will reduce the total number of insulin receptors on all cells where they are present, resulting in lowered overall metabolic efficiency. It has been demonstrated clinically that these persons when given an antioxidant program including vitamins A, C and E along with the enzymes, catalase and superoxide dismutase (SOD), respond favorably in increased health and well-being resulting from, among other causes, an increased response to their own natural insulin.

Fetal insulin receptors are unusual in that they do not undergo down-regulation.[160] This finding is highly significant in relation to live cell therapy. Neglecting antigenicity for the moment, the administration of adult cells rather than fetal cells (for the purpose of obtaining insulin receptors) would result in the eventual internalization and digestion of a certain fraction of these receptors, those that were not recycled to the surface. By administering fetal cells the insulin receptors remain on the surface and are not internalized and down-regulated, thus providing a more long-lasting response to the administration of live cells for this purpose.

Increase in Receptor Number with Differentiation

Although not a means whereby insulin action is normally regulated there has been noted a correlation between an increase in receptor number per cell and an increase in the state of differentiation. Preadipocytes (immature fat storage cells) show a dramatic increase in the number of cell surface insulin receptors during differentiation. T-lymphocytes also acquire insulin receptors upon activation by mitogens (representing a form of differentiation or development).[156]

Following differentiation adipocytes display a 10- to 20-fold rise in insulin receptor level. This increase in receptor number was shown to be due solely to a 20- to 40-fold increase in the rate of receptor synthesis.[156]

Conversely, in hepatoma cells it was shown that specific insulin binding was lower in dedifferentiated cells than in partially differentiated and differentiated ones.[172]

In another study preadipocytes prior to differentiation have virtually no response to insulin in relation to oxidation and glucose uptake. During differentiation these cells acquire the capacity to respond acutely to insulin. Brief exposure to insulin markedly stimulated glucose uptake rates and oxidation to carbon dioxide. The fractional occupancy of receptors required for maximal stimulation of glucose transport into the cell by insulin decreased substantially during the course of differentiation. Thus, it appears that the increased sensitivity of these cells during differentiation is due to both an increase in the number of insulin receptors and their coupling to the glucose transport system.[156]

These findings have a direct bearing on the explanation of results obtained from a type of cancer therapy involving the administration of high dosages of insulin. In this therapy the patient is first fasted. It will be seen below in the discussion of the effect of diet on insulin receptor levels that fasting increases the receptor level in those who are deficient.[153]

Following the fast (and increase in receptor level) the patient is given an excessive dose of insulin sufficient to lower the blood glucose level to approximately 25, almost resulting in coma. Following this treatment the patient is found to have greatly increased ability for drug uptake. Amygdalin (laetrile) or other therapeutic drugs are then administered with greatly enhanced uptake and response.

The fast increases the insulin receptor level which, in turn, increases the sensitivity of cells to insulin leading to greatly increased rates of glucose, amino acid and drug uptake.

Effect of Diet on Insulin Binding

Insulin binding to adipocytes was markedly decreased in rats fed a high fat diet. This was associated as well with a decrease in insulin-stimulated glucose oxidation. The same study showed that lower binding and lower insulin-stimulated glucose oxidation was seen in rats fed a diet high in saturated as compared with unsaturated fats.[153]

Another study showed that excessive intake of both sucrose (table sugar) and fat caused a reduction in insulin binding while a low sucrose diet led to increased insulin binding to monocytes.[154] Diets high in saturated fat caused a 50% reduction in receptor numbers as well as insulin resistance (to be described below).[158]

The preferred divalent cation for the insulin-dependent protein kinase reaction (phosphorylation of the receptor) is manganese.[159] Excellent dietary sources of manganese include wheat bran and orange marmalade.

The Insulin Receptor and Disease

A great variety of human disorders is associated with receptor defects.[160] One example is myasthenia gravis, resulting in antibodies to the acetylcholine receptor. For antibodies to develop, the receptor must have been modified in such a manner that the immune system recognizes it as foreign. Since lipoprotein receptors regulate the disposal of cholesterol a reduction in receptor number may lead to hypercholesterolemia and atherosclerosis. Drug therapy

has been used in the treatment of these two problems to increase the number of receptors.[160]

In Graves disease a defective receptor for thyrotropin leads to the production of antibodies against it, similar to myasthenia gravis.[160]

In several diseases related to the insulin receptor it has been shown that the receptors are normal but that there are fewer numbers of them.[155] In other insulin-related diseases antibodies to the insulin receptor are present, inhibiting the binding of insulin.[152]

Insulin resistance is defined as "a state in which greater than normal amounts of insulin are required to elicit a quantitatively normal response." Receptor studies in several of these resistance states have demonstrated that changes in both receptor concentration and affinity are present.

Several diseases concerned with defective insulin receptor binding are described below. A "post receptor defect" is one that does not involve the insulin receptor itself but some biochemical process following the binding of insulin.

Obesity

Hyperinsulinemia, insulin resistance and a reduced concentration of insulin receptors are well recognized features of obesity. Among obese patients there is found a spectrum of receptor and postreceptor defects. Decreases in insulin binding result entirely from a decrease in receptor concentration. The remaining receptors have normal affinity for insulin. The obese patients with the lowest binding and the greatest receptor loss have the greatest insulin resistance.[152,155]

After insulin-resistant obese patients dieted for several weeks their plasma insulin levels fell and insulin binding to both monocytes

and adipocytes increased. The increased binding was again due solely to an increase in receptor concentration.[152]

Diabetes mellitus

Not all diabetes patients exhibit insulin receptor defects, however heterogeneous defects of insulin receptor function occur. In diabetes with impaired glucose tolerance there is seen a decrease in the concentration of insulin receptors in adipocytes while the affinity of these receptors was normal.[155]

Type 2 diabetic patients appear to suffer from a postreceptor defect. Patients with a more severe insulin resistance display a post receptor defect in addition to the receptor defect.[155] In the treatment of obese type 2 diabetics fasting for two to three days improved insulin sensitivity.

Some type 1 diabetics have a reduced binding capacity while others show an increased binding capacity. In the treatment of obese type 2 diabetics fasting for two to three days improved insulin sensitivity.[155]

It has been shown that exogenous insulin therapy does indeed result in receptor loss.[152] These detailed clinical studies of receptor activity related to the binding of insulin explain at the biochemical level why patients suffering defective insulin metabolism are benefitted by live cell therapy. With the proper choice of tissue having high concentrations of fetal insulin receptors patients may have a receptor deficiency state corrected. As indicated above, some fetal tissue has a greater abundance of insulin receptors than that found on adult cells.

Insulin Receptors and the Immune System

Some researchers have indicated that "fetal pancreatic islets or B cells may be suitable for transplantation and the treatment of diabetes."[162] This statement, made by those who are in no way associated with cell therapy (and may not even accept is as valid) is, in itself, a confirmation of the overall philosophy of this form of therapy. First, it speaks of and implies the lessened antigenicity of fetal cells as compared with adult cells which makes possible the greater acceptance of these cells by the body in conjunction with the utilization of cell surface receptors.

Second, the suggested use of B cells (B-lymphocytes) confirms the long usage of bone marrow by practitioners of live cell therapy. More will be said below regarding still another significance of this class of lymphocyte.

Changes in Insulin Receptor with Age

The influence of aging on the action of insulin was studied using adipose tissue from young (22-30 years) and middle-aged (40-59 years) healthy, normal-weight adults.

Insulin binding was 50% lower in the older than in the younger group due to a decrease in the insulin receptor number. Insulin sensitivity (as judged by glucose oxidation) was 10-20 times smaller in the older subjects.

The basal rate of glucose oxidation in the older subjects was less than half of that for the younger group and the maximum level of insulin-induced glucose oxidation was lower by about 75%.

Apparently aging is accompanied by impairments of the action of insulin on target cells due to alterations at both the receptor and the postreceptor level. These mechanisms and especially the

postreceptor defect may be essential factors in the development of relative glucose intolerance in the aged.[171]

The Insulin-Like Peptides and Their Receptors

No discussion of insulin and its receptor would be complete without including as well the insulin-like growth factors (IGF) and their receptors. The insulin-like growth factors are peptides having molecular weights from 7,000 to 12,000 and resemble insulin in their activity.[164] There are great structural similarities between the insulin receptor and those of IGF.[170] Two separate peptides have been distinguished, known as IGF-I and IGF-II.[162]

Fetal tissues are biologically responsive to IGFs in vitro and are rich in specific cell membrane receptors. Insulin is also bound by IGF receptors but to a lesser degree than IGFs. There is also a weak interaction of IGFs with the insulin receptor.[155]

The level of IGF-I gradually increases in the fetal circulation during fetal life. It is believed that the placenta may play some part in the formation of IGF-II.[162]

There are specific binding sites (receptors) on placental membranes not only for insulin but for both IGF-I and IGF-II. Both IGF-I and II are present in the circulation of the newborn infant and correlate with birth size.[162] There is also compelling evidence that insulin induces the production of IGF-I by the fetal B cell.[162]

It appears that the synthesis and release of peptide growth factors (including IGF-I and II) may be the property of most if not all embryonic and fetal cells. Because the IGF receptors are present on most if not all fetal tissue a recipient of live cell injections will receive these valuable and highly functional receptors along with those for insulin. The administration of these three

types of receptors, those for IGF-I and II along with insulin, explains in large measure the corrective effects of live cell fetal injections in diseases involving insulin.

CHAPTER II — PART V

The Interleukin-2 Receptor and its Significance in Cancer and Infectious Diseases

Introduction

This part, as is true of the foregoing sections in this chapter, is concerned with a particular receptor having great biological significance and of interest to live cell therapists for many years. The receptor to be described is that for a small peptide known as Interleukin 2 (IL-2) having immunological significance.

Recently the attention of the public was directed to research conducted during the past few years related to a new cancer therapy. The treatment involved the peptide mentioned above, interleukin 2, and consists of removing large numbers of white blood cells from the patient, exposing them to IL-2 and returning them to the patient. As a result of this process certain white cells of those treated are activated so as to selectively seek out and kill cancerous cells.

Even though promising and with good results[176,177] the treatment is complicated, has significant side effects, is time consuming and extremely expensive. The patient must be connected to a $30,000 machine for four hours each day with the total treatment lasting one month. The only facility presently providing this service can serve only eight patients at a time.

As adjunctives to live cell therapy, there are many dietary supplements as well as special substances which both stimulate the production of the body's own IL-2 and activate special white blood cells to attack and kill cancer cells.

The presence of IL-2 receptors on both T and B lymphocytes indicate that fetal thymus, spleen and bone marrow are appropriate

tissues for the stimulation of this important aspect of the immune system. The umbilical cord is also implicated as a producer of interferon which, as we shall see below, stimulates the production of IL-2.

Even though this recent medical discovery points a finger at the use of these specific tissues as sources of IL-2 receptors, practitioners of live cell therapy have long known the value of these same tissues in treating cancer and immunological diseases. The presence of IL-2 receptors on fetal thymus cells has been demonstrated.[178]

When medical researchers publish the results of their work in recognized scientific journals the use of live cell administration is always described as "transplantation" rather than "live cell therapy" as used by the proponents of this technique. Conversely, practitioners of live cell therapy could also (justifiably) refer to live cell injections as "transplantation." An example of the former is the use of fetal brain tissue in the treatment of Alzheimer's disease, a practice also conducted by live cell therapists.[179]

Interleukin 2

The technique briefly mentioned above, related to the activation of certain white blood cells by IL-2 to become killers of cancer cells, has long been known. It was the recent commercial availability of large quantities of IL-2 that made possible the reported clinical trials of this technique. There will be described below a detailed account of adjunctive therapy which accompanies the use of appropriate live fetal cell injections including bone marrow, thymus, umbilical cord and spleen.

In the culture of lymphocytes (a group of white blood cells) it was noted that a factor was released into the medium which

resulted in their proliferation whenever stimulated by antigen. Subsequent work showed that IL-2 was produced by T-cells only if first stimulated by another factor, interleukin 1 (IL-1) (produced by macrophages), and antigen.[180] Both IL-1 and IL-2 are known as lymphokines.

Human IL-2 is a single polypeptide chain consisting of 133 amino acids containing one intrachain disulfide bond and having a calculated molecular weight of 15,420. IL-2 binds to a specific receptor on the outer surface (plasma membrane) of T cells and initiates a signal for DNA replication and subsequent mitosis.[181] Only that fraction of T cells previously sensitized to the particular antigen employed will synthesize IL-2 receptors. Although all subclasses of T cells have been shown to release IL-2 (under proper conditions), helper T cells are the major source.[180] Once released, IL-2 promotes the proliferation of any receptor-positive T cell (see Fig. 24).

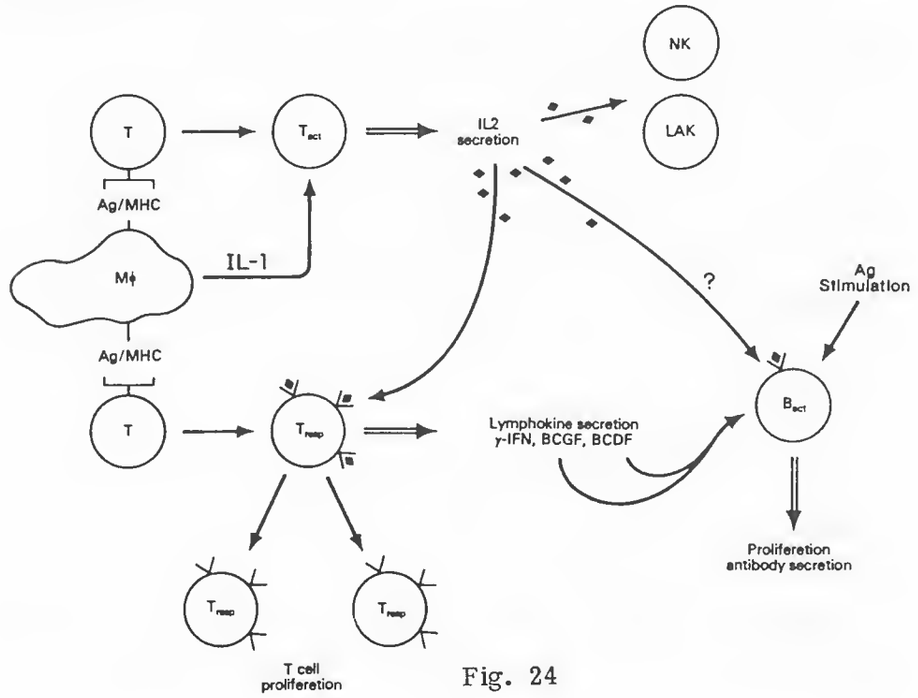


Fig. 24

Activators and Inhibitors of Interleukin-2

Staphylococcal protein A, isolated from staphylococcal cell cultures, induces the formation of IL-2 in human peripheral blood mononuclear cells.[182] The activity of this protein may have some similarities to that of another preparation from staphylococcus known as "staphage lysate," presently being used in cancer therapy. The stimulation of IL-2 by these preparations leads to the activation of "natural killer" cells (described in detail below) which selectively attack and kill cancer cells.

Spermidine (a polyamine found in fish eggs, caviar) enhances the production of IL-2 in cell cultures thereby affecting cellular immunity.[183]

The anti-cancer drug, isoprinosine, potentiates the production of both IL-1 and IL-2 in human monocyte cell cultures.[184]

Hydrocortisone inhibits the proliferation of T cells by causing the IL-2 producing cells to become unresponsive to IL-1 and therefore unable to synthesize IL-2.[185]

Spleen cells, when oxidized by periodic acid (H_5IO_6), produced IL-2. The oxidation of the membrane generates organic carbonyl groups (CO) which serve as triggers for subsequent IL-2 production.[186] It appears that the carbonyl groups arise from the oxidation of cell surface sialic acid. Treatment of spleen cells with neuraminidase (removes sialic acid) prior to periodic acid oxidation inhibited IL-2 production by 84%.[187]

Presumably, any of a number of similar oxidizing agents providing atomic oxygen would yield the same result. A substance consisting of oxygen bound to halogen known as C3 (Dioxychlor) is available for oral use.[248]

Ongoing research with injectable C3 at the American Biologics Hospital in Mexico is showing encouraging results.

Interleukin-2 and Disease

Spleen and lymph node cells from autoimmune mice shown an age-dependent loss in the ability to produce functional IL-2. These results indicate that the immunoregulatory abnormalities of autoimmune mice may be due in part to IL-2 deficiency.[183,189]

When separated by either acrylamide gel electrophoresis or isoelectric focusing, IL-2 separated into multiple peaks of activity. These differences in molecular size and charge were shown to result from differences in sialidation (the attachment of sialic acid to the terminal ends of polysaccharides).[190]

In contrast, IL-2 prepared from a human T-leukemia cell line was uniform in charge and size and indistinguishable from the normal form of IL-2 following removal of sialic acid by neuraminidase.[190] This result implies that the sialidation process in leukemia is defective.

The autologous mixed lymphocyte reaction (AMLR) was significantly deficient in patients with both AIDS (acquired immune deficiency disease) and ARC (AIDS related complex). The in vitro addition of IL-2 enhanced the AMLR to the baseline level of control subjects in most patients in the ARC group but only in 4 out of 15 patients in the AIDS group.[191] Ongoing research with C3 (Dioxychlor) is showing promising results at the American Biologics Hospital in Mexico.

Cytotoxic Cells — Origin and Activity

Cytotoxic cells are specialized white blood cells which are capable of killing other cells under special conditions. The mechanisms by which this action occurs have been the subject of

considerable research over the past 10-15 years. One such cell is the cytotoxic T lymphocyte (CTL) while another is the "natural killer" (NK) cell. Both of these cells kill other cells only under certain specific conditions to be described below. Cytotoxic cells are large lymphocytes with an indented, kidney-shaped nucleus, having a high cytoplasm to nucleus ratio and containing specific granules in the cytoplasm.[192]

In experimental animals, evidence indicates that NK cells originate and, at least in part, differentiate in the bone marrow. However, the exact lineage of these cells is not known with certainty. Data from cancer patients who have undergone irradiation indicate that mature NK cells might be relatively short lived and radiation resistant. In rodents the life span is approximately two weeks.[192]

Mature NK cells from human peripheral blood can be induced to grow in the presence of IL-2 which can also induce generation of NK cells from thymocytes or from peripheral white blood cells lacking the surface markers or mature NK cells (precursor cells).[192]

NK cells have several functions in the body, one of which is to kill virus-infected cells at an early stage of infection.[192] Another is to selectively recognize and kill cancer cells, the recognition being based on the less differentiated state of cancer cells compared to normal cells.[192] More will be said about this selectivity below. It has been found that up to 80% of human large granule-containing lymphocytes can function as NK cells and that all normal humans have these cells.[192]

Mechanism of Action of Cytotoxic Lymphocytes

A great effort has been made in attempting to define specific structures on the surface of target cells that NK cells recognize.

Although this research is not yet complete, several aspects of this selectivity have been found. Susceptibility of target cells to NK cell lysis increases with a decrease in cell surface sialic acid.[193] Of even greater importance is the discovery that one recognition marker may be the receptor for the iron-transporting serum protein, transferrin. It was found that this receptor was present to a high degree on cells that were susceptible to NK cell lysis, namely, fetal cells, including thymus, liver and fibroblasts.[193,194] Transferrin is a requirement for proliferating cells including fetal cells and cancer cells. From these observations it has been suggested that regulation of normal cells may be the primary function of NK cells with surveillance against cancer being a coincidental by-product of the principal purpose.[193] A primary purpose of NK cells may be to control primitive, embryonic or poorly differentiated cells.[193] More will be said below regarding the deficiency of NK cells in cancer patients and the significance in live cell therapy of NK cell attack on fetal cells.

The first step in NK lysis of tumor and other cell types involves binding to the target cell through specific cell surface markers.[195] Following lysis the NK cell is capable of detaching and repeating the process with other cells. This phenomena has been termed "recycling," and has been found to be defective in various immunological diseases as well as cancer.[192] This aspect of NK cell activity will be described in greater detail below in relation to the action of interferon (see Fig. 25).

At the biochemical level there are at least two completely independent basic mechanisms of action whereby NK cells lyse and kill target cells. The first mechanism of action is related to the large granules found in NK cells and other large cytotoxic lymphocytes. These granules are released following binding to the target cell and consist of protein subunits (cytolysin) of a ring which forms

from the linking together (polymerization) of the subunits. The isolation, biochemical and functional characterization of granules from NK cells and other cytotoxic lymphocytes permits the beginning of an understanding of lymphocyte-mediated cytolysis at the molecular level.[196]

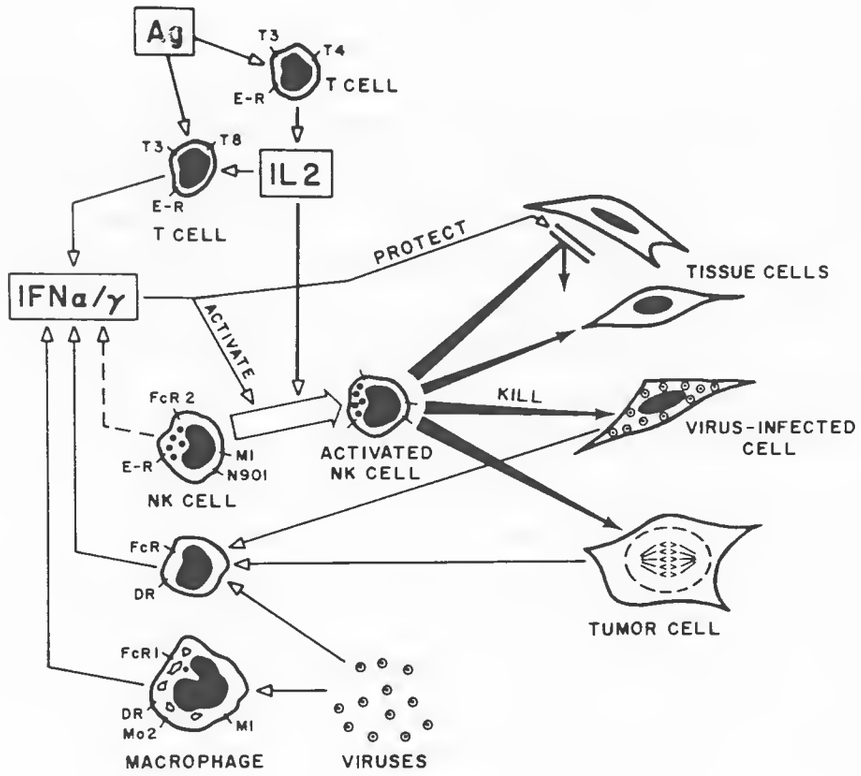


Fig. 25

The rings formed by the polymerization of the subunits stack to form short cylinders 15 nm in diameter which are inserted into

the target cell membrane.[196,197] Presumably, the channel through the center of the cylinder allows the passage of ions (sodium, potassium, calcium) or possibly small organic molecules which increase the ionic permeability and upset the balance of the target cell resulting in cell lysis.[197]

Liposomes are synthetic membranes formed of phosphatidylcholine having the shape of bubbles, smaller than a typical cell. They resemble lysosomes and are formed of lipids, hence the name, liposome. Liposomes formed in a medium containing the water-soluble fluorescent marker, carboxyfluorescein, are also attacked by purified granules of cytolysin. The small cylinders are formed and inserted into the membranes of the liposomes, resulting in lysis and release of the fluorescent dye. Thus, the level of fluorescence is an indication of the extent of liposome lysis through the action of cytolysin.[197]

It has been found that the lytic action of cytolysin is dependent on the presence of both calcium and lipid. In the presence of the selective calcium chelator, EGTA (or EDTA, another calcium chelator), lysis of liposomes was rapidly stopped, indicating an absolute requirement for calcium. This finding has great significance in relation to live cell therapy and the adjunctive use of EDTA as a chelating agent, about which more will be said below.

Cytotoxic lymphocytes are highly specific for particular target cells whereas NK cells will lyse some susceptible tumor targets but not others. The isolated granules, however, show no specificity but will very efficiently lyse all tumor targets.[196] These results suggest that the specificity of NK cytolysis lies in the membrane and not in the basic lysing mechanism (cytolysin).

Another aspect of cytotoxic killing is the degradation of target cell DNA, also calcium dependent. It is believed that the formation of tubular pores (cytolysin) are also responsible for the

DNA degradation, possibly by admitting calcium, a known activator of endonucleases[196] or a small organic molecule also present in the granule.

A second mechanism by which cytotoxic lymphocytes kill cells is related to the production of various soluble reactive oxygen toxic species (ROTS)[87] and their specific delivery to the target cell.[198] The first indication of this was the observation that human NK cells require oxygen for effective killing of tumor cells. Lysis under a nitrogen atmosphere averaged only 52% of the lysis experienced under air. The separate addition of ten different hydroxyl radical scavengers caused a dose dependent inhibition of target cell lysis.[198]

The binding of tumor cells to human NK cells led to the production of hydroxyl radicals within seconds of binding.[198] Plasma membrane fragments from target cells yielded the same results. It appears that NK cells recognize and bind to certain tumor cells and release factors which trigger the release of ROTS from monocytes, an accessory cell in this process.[198]

It has also been shown that large concentrations of superoxide are not required for NK lysis to occur.[198,199] The inability of catalase (destroys hydrogen peroxide) to affect NK lysis makes it unlikely that this member of the ROTS family participates in lysis.[198] Moreover, the addition of singlet oxygen scavengers to human NK cells had no effect on lysis. However, the addition of a singlet oxygen scavenger to murine (rodent family) cytotoxic lymphocytes inhibited lysis by as much as 74% and NK cell lysis by 32%.[198] The particular singlet oxygen scavenger used also reacts with hydroxyl radical making the experiment not definitive. All of the evidence indicates that hydroxyl radical is generated in NK cells by a superoxide-independent mechanism.[198-201] Copper has been shown to increase KN-mediated lysis by as much as 69%

in extremely small amounts.[198,199] KN activity has also been reported to be inhibited by nordihydroguaritic acid, a potent antioxidant having a structure similar to both rutin and quercitin.[199]

These results regarding the mechanisms of target cell killing by NK cells are highly meaningful in relation to live cell therapy. The preservation, as functional cells, of a live cell injection for as long as possible is advantageous to the patient as a source of the biosubstances normally produced by those cells during fetal life as well as during adult life. For example, an injection of fetal thyroid cells will produce the products of the thyroid only as long as the cells remain intact and functional. The valued receptors on the cell surface are transported to various tissues of the body within their own plasma membranes regardless of cellular integrity.

As mentioned above, fetal cells are selectively attacked and lysed by NK cells and other cytotoxic lymphocytes.[193] Because of this it would be of value to the patient to delay the breakdown of these cells by cytotoxic lymphocytes. Through the administration of EDTA, NK cells are not inhibited per se but rather, the action of the cytotoxic granules (cytolysin) is temporarily blocked. Since the EDTA and its calcium chelate are water soluble the temporary arresting of NK lysing activity lasts only until EDTA is completely excreted in the urine.

The use of titrated complexes of antioxidants is also indicated both before and following a live cell injection to prevent the killing action of hydroxyl radical from damaging fetal cells. The HLB Blood Test provides the only practical known means for monitoring excessive toxic oxygen species. All patients should be monitored and given appropriate antioxidant therapy.[87]

The Action of Interferon on Cytotoxic Lymphocytes

Activation of NK cells by virus-infected cells or other antigens induces the production of interferon (IF) by the cytotoxic cell. IF efficiently enhances the cytotoxic activity of NK cells. All three known types of human IF, fibroblast (beta), leukocyte type I (alpha) and leukocyte type II (gamma) increase human NK cell activity although the gamma type is thought to be the most effective.[192] Among other stimulating effects, IF increases the recycling ability (described above)[194] which is defective in various diseases (see Fig. 25).

The cytotoxic activity of NK cells is paradoxically directed against any cell type, including normal tissue cells. However, a lytic effect of NK cells on normal tissue is not seen for several reasons, among which is the continuous inactivation of the NK cells after interaction with normal tissue cells. When an interferon-inducing stimulus appears (virus infection or tumor growth), high levels of IF are produced which activates the NK cells enabling them to lyse almost any cell type. IF protects normal tissue cells from lysis and causes these cells to lose their ability to inactivate NK cells. This further enhances the cytotoxicity of NK cells. In contrast, virus-infected cells and most tumor cells are not protected. There is evidence that the loss of protection is related to a reduction in cell surface sialic acid which has been found on, among other cells, NK cell variants. Treatment of cells with neuraminidase (sialidase) also increases NK susceptibility.[192] The activity of NK cells is then concentrated against the pathologic cell types with IF providing the NK system a high degree of selectivity.[192]

Interleukin II (IL-2) also induces the production of IF (gamma type) in NK cell cultures. It is believed that IF initiated NK cell activation with IL-2 controlling later stages.[202] The interplay here

is complex considering that IF can induce IL-2 receptors on NK cells and that IL-2 can induce the growth and maturation of NK cells.[192] For these reasons it has been suggested that NK cells themselves can function as immunoregulators, controlling their own cytotoxic activity.[192]

Alternations of Cytotoxic Cells in Disease

Tuftsin:

Tuftsin is a naturally-occurring tetrapeptide (consisting of four amino acids) having as a principal function the activation of phagocytic cells including macrophages. (Tuftsin is so-named because it was discovered at Tufts University, Boston.) The amino acid sequence of tuftsin is threonine-lysine-proline-arginine, usually abbreviated Thr-Lys-Pro-Arg.

Tuftsin is a naturally occurring substance becuase it is a part of the immunoglobulin G molecule (IgG) which is freed by the action of two separate enzymes. It is present in the CH2 domain of the F_c (constant) segment of the IgG heavy chain, residues 289-292. An enzyme in the spleen, tuftsin-endocarboxypeptidase, nicks the heavy chain at the Arg-Glu bond between residues 292 and 293. The molecule in this form (called leukokinin) bonds to specific receptors[246] on the outer membrane of a neutrophil, monocyte or macrophage where tuftsin is released by the membrane enzyme, leukokininase, which cleaves the Lys-Thr bond (see Figs. 26 and 27).[203]

If the spleen is removed tuftsin is not set free from the parent IgG and remains inactive with no subsequent stimulation of phagocytic cells. We shall return to the significance of the spleen in relation to live cell therapy below.

Tuftsin has many interesting biological activities among which is the stimulation of neutrophil motility in capillary tubes. In addition, tuftsin stimulates antibody formation following injection into mice. There was an increase in antibody-forming cells in the spleen of more than threefold as compared to controls.[203]

The most interesting in vivo observation has been reported by several groups in this country and abroad. The growth in mice

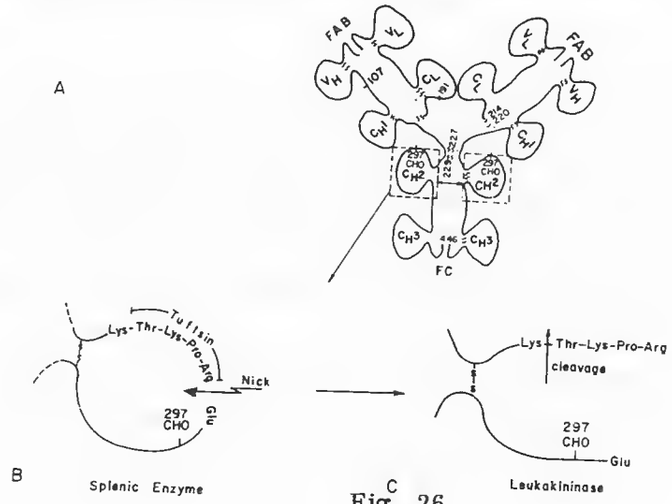


Fig. 26

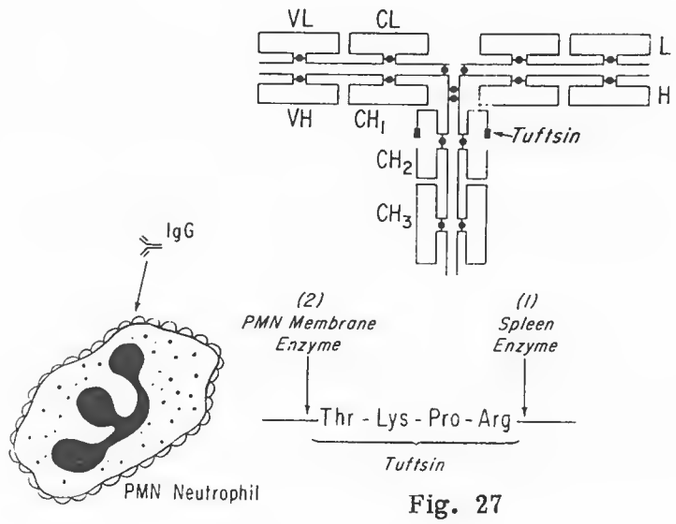


Fig. 27

of several types of tumor cells has been shown to be suppressed or eliminated by treatment with tuftsin. These consist of several types of melanoma, certain strains of leukemic cells, fibrosarcoma and several lymphomas and adenocarcinomas.[203] In another report tuftsin, given to mice after i.p. injection of leukemia L-1210 cells, decreased mortality 50%. All untreated mice died within 11 days of L-1210 cell implantation. Fifty percent of the tuftsin-treated mice survived for more than 30 days.[204]

Tuftsin was found to be active against 3-methyl-cholanthrene-induced transplantable fibrosarcoma in CH3 mice. When tumor cells were injected i.p. into control mice there was a 100% take and tumor was lethal with an average survival of 21 days. When tuftsin was administered a dose range of from 0.2 to 500 micrograms/kg. body weight gave an average survival time of 39 days. Twenty percent of the mice did not develop tumors for a period of 80 days or more.[205] When tumor cells were injected s.c. it was shown that tuftsin retards tumor growth. On day 30 an approximately fourfold difference in tumor volume is seen in comparison to controls.[205]

Cytotoxicity of splenic NK cells from mice was stimulated by tuftsin.[206]

The livers and spleens of control leukemic mice were incapable of checking the growth of bacteria one hour after injection. However, following the injection of tuftsin to the mice the bacterial count fell to 57% of the control (see Fig. 28).[207]

When mice were injected with near-lethal doses of pneumococcus organisms only 10% of the control animals survived while fully 50% survived when given tuftsin.[207]

Bone marrow suspensions prepared after a single i.v. injection of tuftsin showed a pronounced stimulation of NK cell activity.[208]

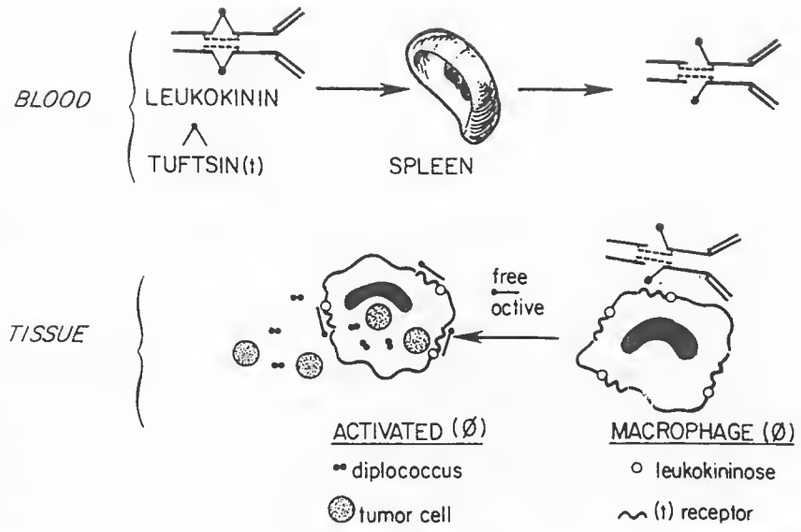


Fig. 28

In mice injected with lymphoma cells, those receiving from 1 to 100 micrograms of tuftsin survived significantly longer than control animals. It is noteworthy that the majority of mice in the groups receiving 10 or 100 micrograms of tuftsin showed no evidence of tumor growth at 150 days post tumor innoculation.[209]

Effects produced by long-term application of the immune modifiers, retinoic acid and tuftsin, on the depressed immune system of 18-month old mice were studied. The effect of these agents on four cells types (cytotoxic T cells, K cells, NK cells and macrophages) possibly involved in antitumor defenses and on the spontaneous tumor development that accompanied advancing age was examined. Common to both agents was an increase of age-depressed tumoricidal activity of peritoneal macrophages. Cytotoxic T cell activity diminished by age was stimulated considerably by both retinoic acid and tuftsin. Histopathologic studies revealed a decrease in the incidence of spontaneous tumors.[210]

Tuftsin was found to have a stimulating effect on depressive behavior in a stress situation in mice by increasing exploratory activity, decreasing fear manifestations and improving avoidance behavior.[211]

Addition of tuftsin to thyroid lobes enhanced TSH (thyroid stimulating hormone) activation of thyroid hormone release.[212]

Results such as these make tuftsin a very attractive agent for immunotherapy against infection and cancer. However, a great deal of caution needs to be exercised when using tuftsin due to inhibitory contaminants found in certain commercial preparations. A high purity commercial product is presently available.

It has been shown that tuftsin deficiency results in immunological insufficiency including greater susceptibility to infection and lessened ability to scavenge tumor cells. The significance in live cell therapy lies in the fact that splenic insufficiency, related to tuftsin release, may be corrected through the administration of fetal spleen cells. Injections of fetal bone marrow cells may be enhanced by prior incubation with tuftsin.[208]

OK-432:

OK-432 is a streptococcal preparation widely used in Japan as an immunopotentiator and antitumor agent. It is a penicillin-and heat-treated lyophilized powder from the Su-strain of Streptococcus pyogenes A3. It has been shown that OK-432 enhances mouse NK activity in vivo and human NK activity both in vitro and in vivo.[213]

When mouse splenocytes were incubated 3 to 4 hours with culture supernatants of mouse thymocytes stimulated by OK-432 a new kind of lymphokine, natural killer cell activating factor (NKAF), was found.[213]

Patients with cancer of the digestive tract (134 cases) were treated with intraperitoneal injections of OK-432. Effusions in the

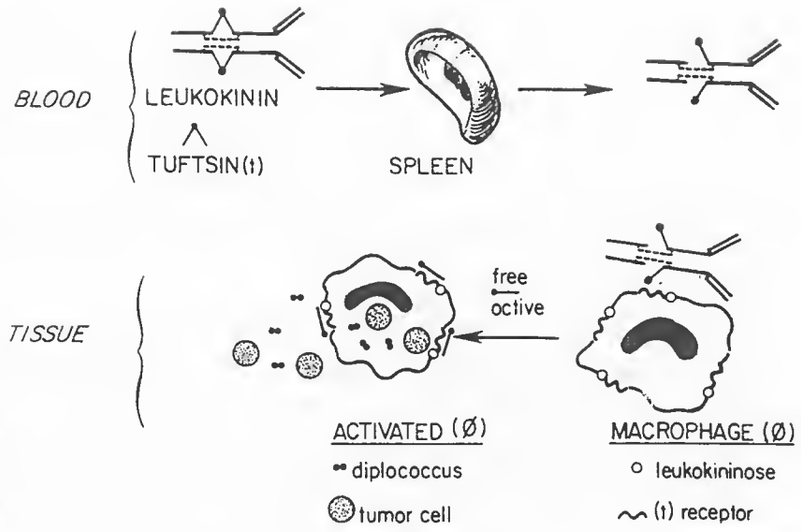


Fig. 28

In mice injected with lymphoma cells, those receiving from 1 to 100 micrograms of tuftsin survived significantly longer than control animals. It is noteworthy that the majority of mice in the groups receiving 10 or 100 micrograms of tuftsin showed no evidence of tumor growth at 150 days post tumor innoculation.[209]

Effects produced by long-term application of the immune modifiers, retinoic acid and tuftsin, on the depressed immune system of 18-month old mice were studied. The effect of these agents on four cells types (cytotoxic T cells, K cells, NK cells and macrophages) possibly involved in antitumor defenses and on the spontaneous tumor development that accompanied advancing age was examined. Common to both agents was an increase of age-depressed tumoricidal activity of peritoneal macrophages. Cytotoxic T cell activity diminished by age was stimulated considerably by both retinoic acid and tuftsin. Histopathologic studies revealed a decrease in the incidence of spontaneous tumors.[210]

Tuftsin was found to have a stimulating effect on depressive behavior in a stress situation in mice by increasing exploratory activity, decreasing fear manifestations and improving avoidance behavior.[211]

Addition of tuftsin to thyroid lobes enhanced TSH (thyroid stimulating hormone) activation of thyroid hormone release.[212]

Results such as these make tuftsin a very attractive agent for immunotherapy against infection and cancer. However, a great deal of caution needs to be exercised when using tuftsin due to inhibitory contaminants found in certain commercial preparations. A high purity commercial product is presently available.

It has been shown that tuftsin deficiency results in immunological insufficiency including greater susceptibility to infection and lessened ability to scavenge tumor cells. The significance in live cell therapy lies in the fact that splenic insufficiency, related to tuftsin release, may be corrected through the administration of fetal spleen cells. Injections of fetal bone marrow cells may be enhanced by prior incubation with tuftsin.[208]

OK-432:

OK-432 is a streptococcal preparation widely used in Japan as an immunopotentiator and antitumor agent. It is a penicillin-and heat-treated lyophilized powder from the Su-strain of Streptococcus pyogenes A3. It has been shown that OK-432 enhances mouse NK activity in vivo and human NK activity both in vitro and in vivo.[213]

When mouse splenocytes were incubated 3 to 4 hours with culture supernatants of mouse thymocytes stimulated by OK-432 a new kind of lymphokine, natural killer cell activating factor (NKAF), was found.[213]

Patients with cancer of the digestive tract (134 cases) were treated with intraperitoneal injections of OK-432. Effusions in the

body cavities disappeared in 76 patients and were reduced in 8 (62.7%). Most patients showed elevated serum protein levels and increased T cells in peripheral blood. In a group of 71 patients, 58 (81.7%) had disappearance or reduction of ascitic fluid which apparently contained tumor cells. The results indicate that this therapy is more effective in patients whose ascitic fluid is positive for tumor cells prior to therapy. For those not receiving OK-432 the survival time of 48 patients was (average) 3.1 months. For those who received OK-432 the mean survival time increased to 10.2 months. Of the 58 who responded well the duration of survival ranged from 2.9 to 36.4 months (average, 15.3 months). This therapy significantly prolonged the survival time and improved immune response in patients who responded well.[214]

In another study immunotherapy with daily intradermal injections of OK-432 over a period of four weeks resulted in quantitative and qualitative effectiveness on impaired cell-mediated immunity, even in many patients with advanced cancer of the stomach or lung.[215] Circulating lymphocyte counts were restored to normal. Survival rates at three and six months following immunochemotherapy using OK-432 and 5-FU (5-fluorouracil) in 40 cancer patients were significantly longer than those of matched control patients given chemotherapy (5-FU) alone.[215]

The mechanisms by which OK-432 augments cellular immunity against tumor cells were studied. Intraperitoneal injection of OK-432 increased both IL-1 and IL-2 production. These results showed that OK-432 induced augmentation of cellular immunity against tumor cells might be due to the so-called "lymphokine cascade reaction" mediated by IL-1 and IL-2.[244]

Following oral administration of OK-432 to mice with intestinal tumors, NK activity of mesenterium lymph nodes increased. It appeared that the activation was mediated by interferons or

IL-2. OK-432 may be clinically useful for the control of digestive tract tumors.[216]

OK-432 enhanced the NK activity of human peripheral blood lymphocytes in vitro and induced the formation of interferons (alpha and gamma). The bacterial preparation also had a direct antiproliferative action on prostate and kidney carcinoma cell cultures.[217]

Vitamins:

Human NK cells showed increased lysis of cancer cells when exposed to retinoic acid (a vitamin A derivative).[218]

Guinea pigs given vitamin E for 3 days showed increased numbers of T lymphocytes in the peripheral blood compared to unsupplemental controls. In mice vitamin E significantly stimulated the activity of NK cells (from spleen).[219]

Minerals:

NK cell activity of normal humans was enhanced by zinc as measured against tumor target cells.[220] For this reason it would appear that zinc is a cancer preventative agent.

A patient with isolated zinc deficiency had lymphopenia (420 lymphocytes/microliter), depressed cell response to stimulus (48% of normal control), an increased number of circulating T-suppressor cells (OKT8) and decreased circulating T-helper cells (OKT4). NK cell activity was 1 lytic unit/10^6 cells (normal of 10-40). Zinc repletion in vivo improved the peripheral lymphocyte count, corrected the abnormal T8/T4 ratio, normalized T cell response and improved NK function. These results are in agreement with those above, indicating that alternations in zinc metabolism may have important implications for human tumor immune surveillance mechanisms.[245]

In contrast, a profound inhibition of NK cell activity was seen following the incubation of spleen cells with high concentrations of zinc.[221]

NK activity of spleen cells from mice was augmented by a single injection of manganese (chloride) administered 1 day prior to testing. Increased cytotoxic activity of NK cells was seen against both NK-sensitive and NK-resistant target cells. NK activity was also enhanced in normally low and intermediately reactive strains of mice. These responses were also accompanied by an increase in circulating interferon levels.[222,223]

In another study the growth of B16-F10 melanoma lung tumors was inhibited in mice injected with manganese one day before tumor challenge. Manganese enhancement of NK cell activity appeared to be mediated by interferon.[224]

The toxic minerals, cadmium, tin and nickel inhibit NK cell activity.[222,225]

Lithium increased both NK cell activity and interferon production in human peripheral blood mononuclear cells.[226]

An oral gold preparation widely used in arthritis therapy (Auranofin) has an effect similar in response and rapidity to that of interferon in increasing the cytotoxic activity of NK cells.[227,247]

Following the oral administration of Ge-132, a water soluble organogermanium compound having the structure $(Ge(O)CH_2-CO_2H)_2O$, a significant level of interferon (gamma) activity was detected in the sera of mice. Ge-132 increased the NK activity of spleen cells at 24 hours and induced cytotoxic macrophages in the peritoneal cavity at 48 hours. Apparently, the augmentation of these immune effects is mediated by the production of interferon.[228,229]

Rats given 0.5 or 2.0 ppm selenium in the drinking water for 10 weeks showed increased NK cell response.[230]

After 7 weeks on a protein deficient diet (4% protein) the NK activity of mice declined to subnormal levels.[232] In mice fed a diet half-deficient in normal levels of all vitamins, spleen NK cell cytotoxic activity fell steeply to low levels after 4-5 weeks and remained depressed thereafter. A similar result was found for IL-2. The ability of spleen cells from the vitamin deficient group to generate IL-2 was markedly depressed after 6 and 9 weeks.[233]

Guinea pigs were fed a diet containing 1% cholesterol and the cytotoxic cell activity was measured. NK cell activity fell rapidly shortly after beginning the diet, decreasing to 25.6% of controls by 2 weeks. NK cell activity was lower in both spleen and peripheral blood of the cholesterol-fed animals.[236]

Additional Activating and Deactivating Factors

CMA-1, a Chlorella extract (from algae) enhanced human NK cell activity.[234]

The administration of a thymic factor (thymostimulin) to mice resulted in considerable augmentation of NK cell activity. The effect was also seen upon administration of another thymic factor (thymosin $alpha_1$).[235]

The cancer therapeutic preparation, BCG (Bacillus Calmette-Guerin), has been shown to induce higher levels of NK cell cytotoxicity.[237]

The drug, methisoprinol (similar to isoprinosine) augmented NK cell activity of peripheral blood mononuclear cells. Overnight incubation of the blood cells, followed by removal of the drug, resulted in significant increases in NK cell activity in all of 17 donors studied. Increase in lytic activity was generally 2-4 fold. The mechanism of action of the drug is to increase the recycling ability of NK cells.[238]

Sodium butyrate (the sodium salt of butyric acid) is a known potent differentiating agent. When human leukemic cells were cultured in the presence of sodium butyrate there was noted a consistent reduction of sensitivity of the leukemic cells toward NK cell cytolysis. Simultaneously, there was noted the appearance of several differentiation markers on the leukemic cells. Following removal of sodium butyrate from the culture medium the leukemic cells recovered their normal NK susceptibility within 4 days. NK cells were seen to be less adherent to sodium butyrate-treated cells than to controls.[239]

In a similar study treatment of K562 tumor cells with sialidase (neuraminidase) was associated with an increase in NK cell susceptibility. Cells cultured in the presence of butyrate had from 3.6-4.0-fold higher sialotransferase activity and were associated with greater amounts of cell surface sialic acid in both sialylglycoproteins and ganglioside (brain) extracts.[240]

The effect of emotional painful stress of different durations on the NK cells of rat spleen was studied. Short term stress (1.5-3 hours) activated NK cell activity whereas long term stress (6 hours) suppressed NK activity.[241] These results implicate prolonged stress as a potential cancer causing factor through the suppression of NK tumor cell cytotoxicity.

Associations between cigarette smoking and the depression of the immune system were investigated by studies of 35 subjects before and 3 months after they had stopped smoking. Those who stopped smoking as compared to those who continued smoking showed an increase in NK activity against cultured melanoma cells.[242] Thus, the presence of carcinogens in tobacco smoke is not the only causative agent in relation to cancer but, in addition, the inhibition of NK tumor cell cytotoxicity.

The NK cell cytotoxicity was markedly enhanced when NK cells were treated with partially purified umbilical cord blood alpha-interferon. Cytotoxicity increased 5-6-fold after treatment with the interferon and the herb, Astragalus membranaceus.[243] This discovery confirms the use of umbilical cord tissue in live cell therapy as an immune stimulant. Through the production of alpha-interferon the cytotoxic action of NK cells is enhanced.

Summary

The significance of the peptide, interleukin 2, has been described with particular emphasis being given as a stimulator in the formation of natural killer cells.

The importance of NK cells in the immunological defense against both cancer and viral infection is stressed.

The factors influencing the development of NK cells are described along with their effects on the activity of these cells.

Certain specific fetal cells, to be employed in live cell therapy, are listed below which have direct application in the process of increasing the population of NK cells in the body.

The live cells and substances listed do not constitute a total program of therapy but are only those described in Part V. (Tissues used: spleen, bone marrow, thymus, umbilical cord, fibroblasts.) Antioxidant pretherapy should be given to minimize Reactive Oxygen Toxic Species (ROTS) activity and monitored by the HLB Blood Test. The use of this test to titrate the dosage of antioxidants administered is a major breakthrough in pre- and post-live cell therapy.

Extracts:

Staphylococcal (including Staphage lysate)

BCG

Thymus extracts

Interferon

OK-432

Vitamins and Dietary Supplements:

Vitamin A

Vitamin E

Rutin, Quercitin

Sodium Butyrate

Spermidine (fish eggs)

SOD, Catalase

Minerals:

Manganese

Zinc

Lithium

Germanium

Selenium

Miscellaneous:

C3 (Dioxychlor)

Isoprinosine, Methisoprinol

Chelation (EDTA)

Avoid:

Calcium and Copper also cadmium, tin and nickel supplementation (during and following live cell therapy)

Cholesterol

Smoking

CHAPTER II — PART VI

Miscellaneous Cell Surface Receptors Related to Live Cell Therapy

Introduction

In the preceeding sections of this chapter the meaning and significance of specific receptors having great significance in both health and disease have been described. There are a multitude of receptors, both known and unknown, which are highly meaningful in the response of cells to extracellular signals and critical to cellular activity.

When an injection of fetal cells is made the recipient is receiving a great variety of meaningful receptors, not only those described in this chapter but many others not yet known. It is not necessary for biologists to name and be aware of certain receptors for the recipient of live cells to receive benefit from them. These receptors described in this part and above are intended only as examples of these remarkable proteins and is not an all-inclusive list upon which research has been conducted.

As a result of the recent research conducted related to cell surface receptors described in this chapter some new fetal tissues are being used by practitioners of live cell therapy for its proposed use.

The intestine contains a receptor (among others) for vitamin D_3. This vitamin (now commercially available separately as a dietary supplement) has been shown to be important in relation to the absorption of calcium. Because of this relationship it is proposed that the use of fetal intestine will be of value in the treatment of osteoporosis and other diseases of calcium maladsorption.

The large number of neuronal proteins that undergo phosphorylation indicates that protein phosphorylation has numerous and varied roles in the nervous system. Those proteins most extensively studied include enzymes involved in neurotransmitter biosynthesis, neurotransmitter receptors, protein kinases, inhibitors of phosphoprotein phosphatase and many others.[258]

Other activities of cAMP-dependent protein kinases (enzymes that phosphorylate proteins) have been amply described in previous sections of this chapter. Brain tissue contains virtually one type of cAMP-dependent protein kinase and one type of cGMP-dependent protein kinase but several types of calcium-dependent protein kinases.[259]

From a study of many types of receptors it appears that regulation of receptor function by phosphorylation is a common and physiologically important property of most if not all receptors. Many types of ion channels (pores in the outer membrane through which ions flow) are regulated by phosphorylation. Some ion channels are neurotransmitter-receptor regulated; that is, the neurotransmitter receptor and the ion channel lie in the same complex. Regulation is achieved by a change in shape of the channel resulting from the binding by the receptor of the neurotransmitter.[259]

A protein of prime importance in neuronal function has been termed, synapsin I, and is found in all neurons. The appearance of synapsin I in the developing fetus coincides with the development of synapses. In the chick cerebellum synapsin I is detected as early as day 17 while in the fetal rat it is detected at day 9.[260]

Synapsin I is bound to the outer surface (cytoplasmic side) of synaptic vesicles, small sacs or bubbles of from 40-60 nm diameter.[261] A "tail" region of the synapsin I molecule is embedded in the membrane of the vesicle. This protein represents approximately 6% of the total protein present in highly purified synaptic

vesicles. These vesicles contain a specific high affinity binding site or receptor for synapsin I. Preganglionic nerve stimulation results in the conversion of about 80% of presynaptic synapsin I from the de-phosphorylated to the phosphorylated form. Additional evidence indicates that synapsin I has a role in regulating the process by which neurotransmitter is released from the nerve terminal.[258,262]

A second neuroreceptor is that for D_1-dopamine (a neurotransmitter) which is found in much greater concentration in the substantia nigra than in the neostriatum. Parkinson's disease is associated with degeneration of nigrostriatal dopaminergic neurons while Huntington's chorea is associated with degeneration of striatonigral dopaminoceptive neurons. The transynaptic effects of dopamine are believed to be mediated by at least two dopamine receptors, one of which, the D_1 receptor, is linked to stimulation of adenylate cyclase (the enzyme forming cAMP). D_1 receptors are found in the neostriatum and the posterior pituitary.[259]

The great success of live cell therapy in treating neurological diseases which do not respond to conventional therapy may be explained, at least in part, by the administration of the receptors for these and other highly significant substances found in neurological tissues.

Using a revolutionary new technique, researchers have injected neurons from fetal rats into the brains of other animals with damaged or defective tissue. Because the brain is an immunologically tolerant site the transplanted neurons form what appear to be normal connections in the host brain and function well enough to alleviate the symptoms of the brain damage.[263] While the technique of injecting live cells directly into the target organ (rather than intramuscularly) differs from the type of therapy described in this text it represents a new and significant variation in previously established protocols of live cell therapy.

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CHAPTER III.

ALPHA-FETOPROTEIN IN RELATION TO LIVE CELL THERAPY

INTRODUCTION

Alpha-fetoprotein (AFP) was first identified in 1956 as a result of electrophoretic studies of the plasma proteins of neonates. The protein migrates electrophoretically between albumin and alpha-globulin and was noted because of its high concentration and, in addition, to its near absence in normal adult plasma.

Interest in AFP was markedly increased with the detection of high serum levels in patients with hepatoma (cancer of the liver) and hepatitis (a viral disease of the liver). Several excellent reviews have been published describing research in this area.[1,2,3,5]

AFP is a single-chain sialylated glycoprotein composed of approximately 580 amino acids and is from 3-4% carbohydrate (including the sialic acid). An estimated molecular weight is 67,000. AFP is a negatively charged protein (acidic) with variations in charge partly caused by the sialic acid content. There has been noted a correlation between the sialic acid content and the immunosuppressant activity of AFP directed against lymphocyte transformation.[1] Those species of AFP carrying the greatest amount of sialic acid were the most inhibitory.[3,4] The biological half-life of AFP in serum is 6 days.[6]

There have been detected no antigenic differences between AFP isolated from tumor-bearing adults and that from fetal sources.[5] Immunization of an animal with its own AFP does not lead to antibody formation.[5] However, antibodies may be developed through immunization with AFP from another species.[1,5] There is enough similarity between human and animal AFP to permit human

anti-AFP made against animal AFP to bind human AFP (cross-reactivity).[1,5] The implication is that animal AFP (arising from fetal cells) given to an adult may lead to the removal of the small amount of AFP present in the plasma of normal adults. Whether this may be advantageous or not is unknown.

AFP IN THE FETUS AND IN PREGNANCY

To return to fetal protein, the synthesis of AFP can be detected in the human embryo as early as 29 days after conception.[6] Near the 14th week of gestation the level rises to 3 mg/ml. in fetal serum, exceeding all other fetal proteins including albumin. At 32 weeks of gestation the level falls to 200-300 micrograms/ml. and to 20-120 micrograms/ml. at term. After birth the level drops sharply. During the first 2 months of life serum levels are about 400 nanograms (1000 nanograms = 1 microgram). After 2 years of age AFP levels are maintained from 3-15 nanograms/ml.[6] As mentioned previously these values may rise as a result of cancer (particularly hepatoma), hepatitis and other non-related diseases. The exact physiological function of AFP remains unknown despite extensive research over many years.[1]

In culturing various types of fetal cells it was found that the largest amounts of AFP were produced by liver cells, smaller amounts by intestinal cells[7] and none by cells of lung, thymus, spleen, skeletal muscle, pancreas or placenta.[1,3] Cultures of human embryonic brain (central nervous system) have also been shown to produce AFP.[8]

A developing fetus is antigenic to the mother since it is a composite of two individuals which are mutually antigenic. This realization is borne out by the fact that transplantations (homografts) between mother and child will reject. It has long been

speculated that (1) the fetus is immunologically accepted during gestation as a result of some form of suppression of the mother's immune system and that (2) birth represents some form of immunologically-caused rejection of the fetus resulting from its antigenicity.

To test the possibility that AFP may be involved in these processes, sera from pregnant mice and mouse fetuses were examined for their effect on the immune system. At midterm of pregnancy there was noted a marked and selective depression of both cellular and humoral response in relation to T-cell (lymphocyte) activity.[9]

A new development relative to the immunosuppression of AFP is the discovery that this fetal protein induces highly efficient suppressor cells.[10] The results of these and similar experiments by several researchers are somewhat controversial.[1,2] It is thought that AFP may function as a "carrier" protein in which certain hormones bound to the protein are transported to certain critical areas. It is also possible that only certain variants or specific forms of AFP are active in immunosuppression.[11] In some of the experiments which have been conducted only crude preparations of AFP were used (for example, whole amniotic fluid) not permitting definitive conclusions to be made.[1]

In another investigation of the effect of AFP on pregnancy rabbit antibodies made against mouse AFP were injected intraperitoneally into pregnant mice. There resulted a 70% incidence of abortion among those injected with the immune sera.[12]

IMMUNOLOGICAL EFFECTS OF AFP

Pregnant mouse serum was shown to suppress antibody synthesis in vitro and was reversed by selective removal of AFP from

the serum. AFP also suppressed lymphocyte transformation by spleen cells from non-pregnant mice. This effect was also eliminated by the selective removal of AFP from the serum.[9]

In another in vivo study suckling mice were treated the first 21 days of life with both amniotic fluid and with AFP prepared from mouse embryos. Antibody production (to sheep RBC) was considerably lower in the treated mice compared to the control.[14]

Splenocytes from normal mice were cultured in the presence of mouse amnionic fluid and AFP for 5 days or more. Antibody production was lowered in addition to a reduction in cell-mediated cytotoxic response to tumor cell cultures.[15]

Pretreatment of macrophages for 24-48 hours with amniotic fluid resulted in a marked reduction in their ability to present antigen to T-lymphocytes. The focus of the suppressive effect of amnionic fluid and AFP is either macrophages or T-lymphocyte-macrophage interactions.[16]

Purified isolates of human AFP (HAFP) from serum, a single hepatoma patient and fetal liver homogenates were extremely potent inhibitors of human lymphocyte transformation in vitro. The relative proportion of a particular varient (HAFP-3) correlated with the capacity to suppress lymphocyte transformation. It appears that an unknown post-synthetic modification of HAFP modulates its immunosuppressive potency[17] and may explain the inconsistent results which have been obtained.

Most of the studies described above were performed in vitro. The extent that AFP is immunosuppressive in vivo[2] has not been established and is part of the ongoing research at the American Biologics Hospital in Mexico.

AFP IN CANCER

As briefly mentioned above, increased levels of AFP were found in hepatoma (liver cancer). The range of AFP concentrations vary a thousandfold, with a lowest value of 50 nanograms/ml. to several mg/ml. of plasma. Higher levels of AFP are found in the large majority of patients who are less than 30 years old but in only about half of those more than 60 years old.[18,19,20] In those having a particular type of liver cancer (hepatoblastoma), seen almost exclusively in early childhood, elevated plasma levels of AFP were found in all.[21]

In vitro cultures of liver tissue obtained from hepatoma patients having elevated AFP levels indicate that this protein is produced exclusively by the liver cancer cells. Normal liver tissue, obtained from the same patient, does not produce AFP.[1,22] Also, only between 10 and 20% of the cancerous liver cells produce AFP.[23,24]

The serum level of AFP often falls temporarily after the surgical removal of a liver tumor. When a disappearance or a decrease in the level of AFP is followed by an increase, this implies a recurrence of the disease.[25]

High levels of AFP are also found in other forms of cancer including teratoblastoma or embryonal carcinoma of the testis or ovary. Out of 27 patients having this form of cancer, 10 had high levels of AFP.[26] This finding has been repeatedly confirmed.[1] Following surgery AFP levels decrease to normal in cases of complete remission but high levels reappear in those who develop metastases. Here again, as with hepatoma, a greater incidence of high AFP values (56%) is found in the young (less than 15 years old) as compared with an older group of patients (15%).[19,20]

In adult animals (rats) given experimental cancer of the liver by feeding specific chemical carcinogens an increased AFP level in the serum may be detected after only 2 weeks. Once detected, the increased AFP level persisted even though carcinogen feeding ceased. Those animals that maintained the high AFP level had a high probability of developing a liver carcinoma. Of those with cancer, 70% had detectable AFP.[27]

Similar findings have been reported in monkeys after the induction of liver tumors by oral administration of a specific nitrosamine (DENA).[28] This carcinogen induces a liver tumor in 82% of treated animals with 60% of the hepatoma cases showing detectable AFP. However, AFP was not found in monkeys given other types of carcinogens.[1,29] The action of nitrosamines is known to lead to the production of free radicals.

When DENA was administered intraperitoneally a hepatoma developed in 95% of the monkeys after a relatively short latent period. In this group 97% had a high serum level of AFP that was detectable about 6 months before any histological evidence of tumor could be demonstrated.[1,29]

Baboons fed a pyridoxine-free (vitamin B6) diet for from 1 to 3 years showed detectable levels of AFP.[30]

HEPATITIS AND LIVER REGENERATION

In a group of 32 patients with acute hepatitis all but one had high serum levels of AFP at one stage of the disease or another. This study showed that the AFP serum level peaks at a time when liver destruction is subsiding and a phase of liver regeneration has begun.[31]

The incidence of abnormal levels of AFP was higher in patients with fulminant hepatitis who survived (85%) than in fatal

cases (39%). The highest levels were found in the most severe cases. If patients were in a coma when AFP appeared in the serum, these patients recovered.[32]

Observations made with experimental animals support the concept that the increased synthesis of AFP in patients with hepatitis is due to liver regeneration.[33,34]

A temporary reappearance of AFP in the serum following treatment of mice, rats and rabbits with carbon tetrachloride (CCl_4) has been noted. In mice the increased synthesis of AFP occurs in nearly all of the animals treated with this organic solvent.[1,35] Interestingly, the detoxification of CCl_4 by the liver is known to involve the production of free radicals. It is interesting, too, to speculate that the derepression (turn-on) of the AFP gene is in some way related to the production of free radicals, considering the action of both carbon tetrachloride and nitrosamine carcinogens mentioned above.[28]

MISCELLANEOUS EFFECTS OF AFP

The persistence of high levels of AFP in the circulation of patients with ataxiatelangiectasia is thought to be related to an abnormality of tissue differentiation.[36] This disease would then appear to represent what might be called a "fetal state" of some tissues in which the genes producing AFP were not repressed in a normal manner.

A particular species (variant) of rat AFP strongly inhibits inflammation caused by a variety of inflammatory substances. Furthermore, this same fraction completely suppresses inflammatory reactions during hepatitis induced by galactosamine (a particular aminosugar).[13] This may represent another example of the regenerative ability of AFP in relation to the liver.

AFP IN RELATION TO LIVE CELL THERAPY

Live cell therapy is the intramuscular injection of live cells from a fetus, usually of bovine origin. One indication or clue as to the significance of AFP in live cell therapy is the requirement (learned by experience) that the fetus not be older than 70% of term. As mentioned aobve the production of AFP peaks near midterm (50%) and declines sharply thereafter. Although not proof of the significance of AFP in live cell therapy, this fact is an indication that AFP may be involved in the regenerative and rejuvenative effects derived from this form of therapy. This belief is further confirmed from the known regenerative ability of AFP in relation to the liver.

There is much evidence given above to indicate that AFP is associated with the turning on of genes (derepression), first in the fetus, in regenerating liver and finally in cancer (cell proliferation) and other diseases representing a state of dedifferentiation or "fetal state."

The relationship of AFP to hepatitis is in agreement with the concept that this fetal protein may function, at least in part, as a gene derepressant. It is well known that viruses act as gene manipulators, turning off some and turning on others. The presence of increased levels of AFP during a crisis of hepatitis always indicates that regenerative processes are underway. These observations indicate that the administration of fetal liver cells may greatly assist in combatting hepatitis as a result of their ability to generate large amounts of AFP, thereby resetting liver genes to their natural state.

In several disease states mentioned above (hepatitis, cancer, for example) higher levels of AFP were seen in younger persons

than in older. This response may result from two causes. It is known that with aging certain genes that have always been turned on are eventually turned off. The agents blocking these genes are apparently unable to be removed through the action of AFP. Also, older persons have been for a longer time under the influence of toxic substances arising from food, body metabolites, smoking, etc. These agents (blocking the AFP gene itself) may not be capable of being removed by AFP leading to the observed results regarding age.

It was mentioned above that when AFP from another species is administered (through synthesis by fetal cells), antibodies are generated against this protein. There is enough similarity between bovine and human AFP to cause the antibodies to also be effective against human AFP as well (crossreactivity). This implies that live cell therapy should be approached cautiously during pregnancy because of the possibility of causing an abortion. It is known that antibodies (IgG) can cross the placental barrier and enter the fetal blood. Anti-AFP would then bind the fetal AFP and, could cause an abortion. This technique, on the other hand, may be advantageous in causing a desired medical abortion.

It has been theorized by practitioners of live cell therapy that, in cancer, the use of fetal cells of the same organ in which the cancer is found (fetal liver for hepatoma, for example) could lead to increased tumor activity. However, cancer is retarded by the use of fetal spleen, placenta and thymus cells, the very tissues which produce no AFP. Ongoing research at the American Biologics Hospital in Mexico with the same kind of fetal cells as the cancer (those that produce AFP) has resulted in encouraging reduction in tumor activity, particularly in liver and lung. Research is continuing in this area. The synthesis of AFP by cancer cells may keep certain critical genes turned on which encourages cancer growth.

AFP generated by bovine fetal cells injected into a patient will result in anti-AFP. These antibodies will cross-react with bovine AFP and neutralize both bovine and human forms. However, the humoral immune system of cancer patients is greatly weakened and may not be able to completely eliminate the combined AFP produced by both the tumor and fetal live cells. Since additional AFP (from some fetal cells) is deliterious to recovery from cancer, it would appear that non-AFP-producing fetal cells (placenta, spleen, etc.) combined with antibodies to AFP would constitute a new treatment for cancer.

MECHANISM OF LIVE CELL THERAPY

The mechanism of live cell therapy may consist, at least in part, of the following steps.

(1) The "targeting" of the injected cells to the particular organ in the body represented by those cells (fetal liver cells migrate to the liver, etc.).

(2) The generation of fetal AFP within the target organ thereby exposing the cells of that organ to high concentrations of AFP.

(3) The turning on (derepression) of genes within that organ's cells which may be blocked or otherwise repressed through the action of toxic substances, viruses, cancer, etc.

(4) The generation of vital cellular factors, for example, hormones, nucleic acid (m-RNA), etc., which are normally generated by younger cells but which have been, for some reason, suppressed in older cells.

(5) The regeneration of these older cells by the vital factors so generated.

This process may be likened to the Oriental dietary practice of eating sprouted beans rather than beans themselves. In sprouted beans certain genes have been turned on permitting the synthesis of vitamins and other vital nutritional factors. Unsprouted beans do not contain these substances because the genes that code for the enzymes which synthesize the vitamins, etc., are turned off until sprouting occurs.

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CHAPTER IV.
CELLULAR EXTRACTS

INTRODUCTION

Because of the permanent nature of binding cell recognition receptors to host cells the effects from live cell therapy are more long lived than those of cell extracts. As mentioned previously, there is a delay in the onset of the long-term response seen in live cell therapy, possibly from weeks to months. During this period a patient may receive temporary benefit from injections of cellular extracts prepared from the corresponding organ.

The extracts described below and their effects are given as representative of the responses of various diseases to cellular extracts. Healthy cells normally produce adequate amounts of these vital substances but with ageing, production diminishes, resulting in disease.

A description of selected cellular extracts and their medicinal effects are given in this text for two reasons. First, live fetal cells injected into the body will continue to produce those substances characteristic for each type of tissue for a limited time. This biosynthesis of limited duration may explain the immediate and short-term response noted following live cell injection (Chapter II).

Second, contact of cell recognition receptors on the surface of host cells with corresponding receptors liberated from fetal live cells of the same organ actuates genes which have long been masked, resulting in the normal biosynthesis of vital cellular substances. Thus, the administration of cellular extracts mimics the long term effects seen with live cells carrying the all-important and vital cell recognition receptors.

Cellular Extracts

Cellular extracts are chemical substances that are removed from specific tissues or organs through the use of extraction solvents, most often buffered salt solutions but may consist of methyl alcohol, carbon tetrachloride, ether or other organic solvents.

Extracts from specific tissues contain vital substances which control either the activities of those cells or other organs in distant parts of the body. When administered in concentrated form these substances have profound physiological and rejuvenative effects.

Some extracts from specific tissues contain substances which have been known for many years (for example, thyroxine from thyroid). However, extracts from many tissues contain physiologically active substances which have never been characterized. An example of a tissue containing active substances which have only recently been characterized is the thymus.

Regardless of the method of extraction, whether by an organic solvent or water, the extracted substances must be dissolved in water prior to use.

RNA

Ribonucleic acid (RNA) may also be considered a cellular extract even though it does not normally leave the cell in which it was formed. Conversely, RNA cannot be incorporated into cells if administered intravenously or intramuscularly as will be shown below.

One problem met by those performing cell culture is to introduce macromolecules into the cells since these substances cannot pass through the cell membrane. Very ingeneous and elaborate techniques have been developed for the purpose of incorpo

rating large molecules into cells. One of these techniques was developed specifically to incorporate transfer RNA (t-RNA), messenger RNA (m-RNA) and ribosomal RNA (r-RNA) into cells. The very fact that such a technique was developed specifically for the three most common forms of RNA indicates that RNA is not normally incorporated into mammalian cells (except for some white cells which are capable of phagocytosis).[19]

In experiments conducted with certain frog cells it was desired to introduce both m-RNA and r-RNA into the cells. This was accomplished by the microinjection of individual cells.[20] If RNA passes through the cell membrane this problem would have been solved simply by introducing RNA into the culture medium and allowing it to pass through the membrane into the cell. (For additional information relating to the passage of RNA through cell membranes see Chapter II, pg. 31.)

LIVE CELL EXTRACTS

Extracts have been made from each of the seven tissues listed below and given to patients for various diseases. Under each tissue is described the method of extract preparation, mode of administration of the extract, purpose of treatment (disease), results and the literature reference. In some of the entries the extract was used in vitro as a part of a research study showing the effect of the extract on cells in culture.

Tissue: Placenta

Disease: Myopic and Senile Chorio-retinal dystrophies (eye) (34 cases)

Method of Application: Aqueous solution, intramuscular injection.

Parameters Measured: Visual acuity, luminous sense, visual field and the electrophysiological activity of the retina.

Results: The parameters measured were improved by the administration of the placenta extract, particularly in the significant improvement in the luminous sense.[11]

Disease: The loss of hair in women (65 cases)

Method of Application: Injections

Results: The overall rate of improvement was 74%. Total recovery occurred in 43% of cases. In postpartum cases the rate of improvement was 91%, recovery, 64%.[2]

Disease: Rheumatic affections (acute rheumatic inflammation)

Extract: Placenta-Lucchini (commercial product)

Method of Application: Iontophoresis combined with catalase and mucopolysaccharidase (enzyme). The treatment also includes intro-articular application in the joints.

Results: Significant clinical improvement in symptoms.[3]

Disease: Bone lesions (fractures)

Extract: Human Placenta

Results: An exorbitant increase in alkaline serum phosphatase, a remarkable atypical intermediate callus phase and an earlier solid healing than was observed with controls.[4]

Disease: Biochemical study to investigate the chemical nature of the ability of placental extracts to stimulate the release of pituitary thyrotropin.

Extract Preparation: Normal human placentas were ground at 4^{o} C, freeze-dried and then pulverized in a food blender. The pulverized tissue was extracted first with carbon tetrachloride (CCl_4) for 4 hours in a Soxlet extractor to remove lipids (fat). The tissue was then extracted by shaking the tissue with methanol (methyl alcohol) at room temperature. Methanol extracts were obtained by evaporation of the methanol.

This method of extraction indicates that the active substance may be either a peptide or a simple organic molecule.

Method of Application: in vitro study.

Results: These experiments confirmed the presence of substantial quantities of materials in placenta which possess throtropin-releasing activity.[5]

Tissue: Thymus

Disease: Juvenile chronic arthritis (3 cases)

Extract Preparation and Method of Application: Details in German (book)

Results: A striking clinical improvement was observed in all three cases. Laboratory data including cellular immunoreactivity normalized in all patients.[6]

Disease: Recurrent herpes simplex (21 cases)

Results: An impressive reduction in both the number and

severity of the recurrences of the infection was observed in patients treated with the extract, both during the treatment period and for up to 3 months afterward. A significant increase in total WBC, lymphocyte count and T-cells was detected after 6 months. In vitro lymphoproliferative responses to herpes simplex virus antigen and natural killer cell activity were also significantly higher.[7]

Disease: Immunodeficiency in children (8 cases)
Method of Application:
Results One child with severe combined immunodeficiency showed marked clinical improvement together with reconstitution of T-cell and B-cell numbers and immunoglobulin production. Two further children showed benefit from the therapy while none of the other children showed any consistent improvement.[8]

Disease: Tuberculosis (in experimental guinea pigs)
Extract Preparation: Text in Russian
Method of Application: Subcutaneous injection
Results: A considerable decrease in the severity of the tuberculosis process.[9]

Disease: Untreated Hodgkin's disease (19 cases)
Extract Preparation: Calf thymus tissue is first minced and extracted with ammonium acetate. This extract is subsequently treated with ammonium sulfate. The precipitate is dissolved in water, subjected to ultrafiltration and the filtrate desalted by column chromatography on Sephadex G-25 and gel filtered on Sephadex G-50. The two active fractions obtained are lyophilized.

Method of Application: intramuscular injection
Results: The mean percentage of peripheral blood lymphocytes forming E-rosettes increased from 47 to 55.7% (normal, 58.9%). The mean PHA stimulation index rose with all three concentrations tested but did not reach normal values. Thymus extract, in vivo, appears to return immunologic competency to a population of untreated patients with Hodgkin's disease.[10]

Disease: Immunodeficiency (24 cases)
Extract Preparation: Text in Italian
Method of Application: Injections
Results: A significant positive response (from 47 to 84.6%) was noticed in patients treated with leucotrophina with ages between 65 and 75 years. No remarkable improvement was seen in patients over 75 years of age.[11]

Disease: Histiocytosis-X. A granulomatous formation with infiltration and proliferation of histiocytes. Characterized by a lack of H2 surface receptors on T-lymphocytes suggesting a suppressor-cell deficiency. (17 patients)
Extract Preparation: Calf thymus tissue from 5-day old animals was homogenized in phosphate-buffered saline (pH7.2) for 5 minutes at 4^{o} C. in a blender. This was centrifuged at intermediate speed for 20 minutes. The supernatant was lyophilized and reconstituted in a smaller volume. This was dialized for 60 hours at 4^{o} C. The dialysate was lyophilized and reconstituted with water. The pH was adjusted to 7.4 and sterilized by passage through a Millipore filter.
Method of Administration: Daily i.m. injections, 1 mg protein/kg body weight.

Results: Seventeen patients were treated with daily injections of thymic extract. Ten of these patients entered complete remission following treatment. Seven of the 17 patients had previously received chemotherapy. Only 2 of these 7 responded to thymic extract. Out of 10 other patients who had no previous chemotherapy 8 responded favorably to thymic extract.[21]

Tissue: Spleen

Disease: Lipid metabolic disorders in systemic connective tissue diseases.
Extract Preparation and Method of Application: Text in Russian. The extract used was Splenin (from spleen).[12]
Results: Considerable improvement was noted in the test animals as compared to the controls.

Tissue: Liver

Disease: Acute liver failure (experimental, in rats)
Extract Preparation: Rat liver was homogenized in a Potter-Elvehjem tissue grinder. The crude homogenate was passed through surgical gauze. This was centrifuged for 10 minutes at 1000 g followed by centrifuging the supernatant at 100,000 g for 1 hour.
Method of Application: intraperitoneal injection
Results: In rats the liver cytosol fraction acted by stimulating the rate of endogenous liver regeneration and hepatocyte proliferation.[13,14]

Tissue: Brain

Disease: Radicular compression (concussion) (71 patients)
Extract Preparation: The extract consisted of bovine cerebral cortex gangliosides.
Method of Application: Daily doses of 20 mg for the first 15 days and 10 mg thereafter for a total treatment program of 20-25 days.
Results: The statistical analysis showed that the reflexologic parameters tested revert to normal quicker in the patients treated with gangliosides than in those not receiving such therapy.[15]

Tissue: Parathyroid

Disease: Postmenopausal osteoporosis (12 cases)
Extract Preparation: Commercial preparation (Eli Lilly Co., Indianapolis, Ind.)
Method of Application: Intramuscular injection
Results: Parathyroid extract (PTE) was administered to stimulate the enzymatic conversion of 25-hydroxyvitamin D to 1,25-dihydroxyvitamin D which is the major hormonal regulator of calcium absorption. After PTE administration, serum calcium concentration and serum 1,25-dihydroxyvitamin D increased significantly in all patients.[16]

Disease: Hypoparathyroidism (2 cases)
Extract Preparation: Commercial preparation (Eli Lilly Co., Indianapolis, Inc.)
Method of Application: Intramuscular injection
Results: Parathyroid extract increased the serum 1,25-

dihydroxyvitamin D* level in both patients (see previous disease) to highly significant degrees.[17]

Tissue: Pituitary

Disease: Biochemical study to indicate chondrocyte proliferation by pituitary extract (PE).

Extract Preparation: Human pituitary glands were homogenized in 5% formic acid, in 0.1M sodium bicarbonate, pH 8.5 The formic acid and $NaHCO_3$ homogenates were stirred 2 hours in the cold (4° C.) and centrifuged at 100,000 g for 1 hour. The supernatant was passed through cheesecloth to remove any residual lipid material and the pH was adjusted to 7.4. The supernatant was concentrated by ultracentrifugation. The $(NH_4)_2SO_4$ was removed by repeated dilution and concentration of the supernatant with .01 M Tris-HCl buffer, pH 8.5.

Results of in vitro assays: Following application of PE to chondrocyte cultures there resulted in a 10-fold increase in cell number. It appears that the pituitary may contain yet undiscovered hormones which stimulate different kinds of cells to grow.[18]

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CHAPTER V.

THE PREPARATION AND PRESERVATION OF CELLS FOR USE IN LIVE CELL THERAPY

INTRODUCTION

There are many techniques for the isolation of tissues from a fetus leading to the eventual cell suspension used clinically in the practice of live cell therapy. Each facility engaged in this process has developed methods (some proprietary) for achieving the desired objective, namely, the removal of specifically chosen cells, tissues and organs from a selected fetal animal under sterile conditions with preservation techniques to insure viability when used clinically. Although temporary techniques of live cell extraction, preservation and use have been developed over the last hundred years or more, in the last ten years significant advancements in technology have improved quality control of sterility, antigenicity, preservation and optimum cell viability. Millions of research dollars are being spent in live cell therapy technology in such diverse areas as cell culturing and monoclonal antibody production. It is not within the scope of the biochemical basis of live cell therapy to give an in-depth dissertation on cell preservation techniques, however, a preferential overview with some of the recent research developments will give the reader an appreciation for this rapidly developing technology.

ISOLATION OF FETAL ORGANS

There will be described below one process for the isolation of fetal organs for use in live cell therapy. The chorion enclosing a bovine fetus is removed by cesarean section from a pregnant

female. The age of the fetus is known with certainty by selective breeding and should be no more than 50% of term. The biochemical rationale for this age limitation is described more fully in Chapter II.

The surface of the chorion (fetal sac) is typically bathed with tincture of iodine providing some degree of sterility before removal of the fetus. Sterile technique is used in both the removal of the fetus and subsequent removal of organs.

The fetus is rinsed with iodine solution followed by Hartmann's solution. The placentas are then removed and kept for further processing. Even though not a fetal part, this tissue is extensively used in live cell therapy.

Organs and other parts of the fetus are removed in a specific sequence, beginning with the removal of the skull and proceeding throughout the entire length of the body.

In addition to specific organs other tissues are collected including, but not limited to, umbilical cord, muscle, intestine, cartilage, neurological tissue and synovial fluid from the knee joint.

Organs and tissues which have been removed are kept in covered, sterile plastic cups until ready for processing. Each tissue is placed in a mortar along with Hartmann's solution (a physiological nutrient medium) and gently ground to form a cellular suspension. The suspension is withdrawn into sterile, non-pyrogenic syringes and stored briefly until further preserved by freezing (described in greater detail below). Microscopic examination reveals large clumps of cells rather than free, individual cells.

An aliquot is taken of each tissue type and tested for antigenicity, contamination, as well as viability. The analytical procedures for making these tests, many of which have been developed within recent years, are available in the technical literature and are not within the scope of this presentation. However,

anyone anticipating performing cell extraction would have to become proficient in modern analytical techniques.

TISSUE DISSOCIATION

Other techniques for tissue dissociation will now be described which give superior results.

(1) A fine mesh stainless steel screen wire (of a few thousandths of an inch mesh) is cut to a circular shape and welded into the bottom of a stainless steel surgical pan (with a hole cut in the center). Using sterile technique, tissue is gently pressed through the screen wire and scraped off the bottom. The cells are suspended in a physiological nutrient medium.

(2) A Potter homogenizer for cell dissociation consists of a Teflon pestle or rod with rounded end mounted on a 1/4" steel shaft. The steel shaft is mounted in a rotational press. The tissue to be dissociated is placed in a tight-fitting test tube especially made to have a specific clearance (a few thousandths of an inch) with the pestle. Tissue is placed in the glass tube along with a small amount of nutrient medium. The press is turned on and the glass tube with the tissue is slowly brought higher and higher, forcing the tissue into the clearance between the pestle and tube, fragmenting it into individual cells. The tube is slowly lowered and the process repeated many times. The glass tube is constantly submerged in an ice water bath to prevent heating. The entire assembly is autoclavable and kept sterile during the operation.

CELL PRESERVATION

Once fetal cells have been isolated they must be either used immediately or preserved in one manner or another for future use.

The immediate use of isolated fetal cells, however, is a risky practice, bypassing quality control analytical procedures for antigenicity, sterility, contamination and viability. Two techniques which may be used to preserve cellular material are (1) freeze-drying (lyophilization) and (2) cryopreservation. Each of these cell preservation methods will be discussed separately.

Freeze-Drying

Since one fundamental requirement in the preparation and use of fetal cells is sterility it becomes apparent that some method of preservation in which bacteria cannot grow must be quickly employed following isolation. It would also be advantageous to be able to transport fetal cells over relatively short periods of time by air, ready for injection upon arrival.

Both of these requirements are met by the freeze-drying process briefly described here. Freeze-drying is a method of preservation which is applicable to many commodities and products as well as mammalian cells. The material containing water which is to be preserved is first frozen and then subjected to a high vacuum. While under vacuum ice crystals are sublimed, that is, water in the solid state (ice) passes directly to the gaseous state (water vapor) without passing through the intermediate liquid state (water). The product is an anhydrous powder which cannot support the growth of microorganisms because it contains little or no water.

While the absence of water from freeze-dried cells fulfills the two requirements mentioned above (inability to grow bacteria and the possibility for long-distance transport) there are three major disadvantages to the practice of this technique as a means of preserving live cells for use in therapy.

(1) The significance of the immunological response in live cell therapy is described in detail in another part of this text

(Chapter II, Part I). It is indicated that if the immunological response to the injection of foreign material is too great partial or in some cases complete loss of any expected benefit from the therapy may be experienced. During the freezing process required for lyophilization and the subsequent removal of water by sublimation, cells are ruptured with the subcellular contents spilling out and being made available for contact with macrophages, those white cells engaged in the transfer of information to the lymphocytes where antibodies are synthesized. Simply contact of foreign protein and polysaccharides (antigenic material) with macrophages stimulates the inflammatory response and the production of antibodies against these substances. By breaking open cells through freeze-drying many more antigenic substances are allowed to contact the immune surveillance system sooner than would otherwise occur if whole cells were injected. If the response to this antigenic insult is too great, the inflammatory processes are brought into play at the injection site which may result in the total digestion and breakdown of the cellular material and forcing the use of antiinflammatory agents. By attracting additional white cells into the area (chemotaxis) of injection the inflammatory process is esculated to the point where no macromolecules remain intact.

(2) Also described in Chapter III is the significance in relation to the inflammatory response of a specific substance synthesized by fetal cells, that of alpha-fetoprotein. It was pointed out that this protein has the ability to suppress certain aspects of the immune system[1-5] and may be instrumental in preventing the rejection of the fetus by the mother during gestation. This protein is produced only by living cells, not by cells that have been ruptured and killed by an improperly executed freezing process and/or dehydrated by lyophilization. If alpha-fetoprotein is not produced, resulting in an excessive inflammatory response, the optimum bene-

ficial effect from live cell therapy will not be realized.

(3) It has been indicated in Chapter II, Part I that the value of receiving injections of fetal cells may lie in specific macromolecular proteins known as cell recognition and adhesion receptors (lectins and glycoproteins). When these macromolecules contact corresponding receptors on the organ of the recipient highly meaningful signals are sent into the cell which are capable of influencing the activity of the genetic material (DNA).[6] By this process the cell is restored to a more normal state through the masking and unmasking of specific genes (DNA) which, in turn, modulates enzyme synthesis and other cellular activity.

It has been amply demonstrated that cell recognition receptors, highly significant in live cell therapy, are inactivated by lyophilization (freeze-drying). Although using standard viability techniques, research conducted in England by the Burroughs-Wellcome Co.[7] showed that mouse spleen cells are capable of synthesizing antibodies (protein synthesis) after being freeze-dried and rehydrated provided a cryoprotective agent is used (in this research, polyvinylpyrrolidone, PVP). These cells following rehydration were shown to be 87% viable by dye exclusion techniques (trypan blue) even though the receptors on these cells have been inactivated. In this test those cells which are alive appear colorless because of their ability to exclude the dye from entering the cytoplasm. Dead cells are stained blue because the dye is able to pass the plasma membrane which is inactive.

Phytohemagglutinin (PHA) is a lectin (protein) isolated from plants which activates lymphocytes as measured by the uptake of tritiated thymidine (a radioactive constituent of DNA). Receptors normally present on the surface of lymphocytes are bound to PHA through carbohydrates found in the receptor. For this to occur the receptor must be in its natural, active state, that is, undamaged.

PHA may be covalently bound to small beads formed from a particular insoluble polysaccharide (Sepharose, Pharmacia Fine Chemicals) and in this state, is said to be "immobilized." If a substance has a specific binding affinity for PHA it will bind to the bead until washed free with specific salt solutions.

Spleen cells and lymphocytes which have on their surfaces receptors showing a specific binding affinity for PHA will bind to Sepharose beads carrying this bound lectin. When normal spleen cells (or lymphocytes) and the same cells which have been freeze-dried and rehydrated are stirred and incubated separately with PHA-Sepharose beads, it is found that 60% of the freeze-dried cells have damaged receptors and do not bind to PHA.[7]

When a similar lectin (Con-A) is also permanently attached to Sepharose beads, freeze-dried cells are 55% damaged as revealed by their binding ability.[7] When PHA was administered to lymphocytes that had been freeze-dried and reconstituted they failed to incorporate tritiated thymidine indicating that the cell surface receptors were damaged by freeze-drying.[7]

When normal mouse spleen cells are incubated with sheep red blood cells (RBC) they form what are known as "rosettes" which are simply individual spleen cells surrounded by clusters of RBCs. The formation of spontaneous rosettes between these two cell types indicated that there are cell recognition receptors on their surfaces which have an affinity to each other (lectin-glycoprotein complex).

When mouse spleen cells which had been freeze-dried and rehydrated were incubated with sheep RBC a 94% reduction in the number of rosettes was seen. In one experiment no rosettes were seen on 9 replicate tubes while in another experiment only 50 rosettes were seen per 1 million cells (normal, 500-1000 rosettes/million cells).[7]

In these experiments a special slow rehydration technique was employed in which the cells reincorporated water at the temperature of melting ice (0° C.). This technique (employing dry ice) is not practical for a practicing clinician of live cell therapy. However, these experiments forcefully demonstrate the inactivation of cell recognition receptors by freeze-drying.[7]

Other proteins have also been noted to suffer damage from freeze-drying. Hemocyanin, the copper-containing protein found in some lower sea animals, corresponding to hemoglobin, lost the ability to bind oxygen and no longer turned blue when exposed to air following freeze-drying.[8]

When the enzyme catalase is freeze-dried and rehydrated several types of products are formed. In one the individual identical subunits which make up the complete molecule were dissociated and had no enzyme activity following rehydration. It is believed that all of the subunits that comprise various forms of catalase isolated from many species are required for enzyme activity. The four subunits of catalase have been shown by X-ray crystal analysis to form a barrel through which the substrate (usually hydrogen peroxide) passes. If the subunits cannot associate (as a result of freeze-drying) the barrel does not form and no enzyme activity is seen.[9]

The realization that cell recognition and adhesion receptors found on the surface of fetal cells are inactivated by freeze-drying has highly significant implications in the practice of live cell therapy. As stated above, the process of freeze-drying offers several attractive solutions to problems encountered in the preservation and distribution of live cells intended for therapy. However, some of the biochemical modes of action of live cell therapy as set forth in this text will be adversely affected (see Chapter II). The process of freeze-drying will diminish the intended purpose of

live cell therapy through inactivation of specific membrane receptors found on the cell surface.

Cryopreservation of Cells

The solution to the dilemma presented above lies in extensive research carried out over many years in efforts to develop a process for freezing cells which, following thawing, will remain largely viable. As a result of this research it was shown that many factors are involved, not only the freezing process itself but also the manner in which the cells are thawed. It is entirely possible to practice a freezing technique resulting in viable frozen cells which are then killed by an improper thawing procedure. Both of these steps are critical for ultimate cell survival.[10]

One of the major damaging factors to be encountered in attempts to preserve the viability of cells is the formation of intracellular ice crystals (ice in the cytoplasm of cells). Unless measures are taken to inhibit the growth and enlargement of such crystals the outer or plasma membrane of the cell will be ruptured leading to cell death. Cells are normally kept in a "physiological" salt solution in which the concentration of salt is equal to that within the cell. As the temperature of the cell suspension is lowered the formation of ice in the external fluid increases the salt concentration simply by removing water (as ice). Increased salt concentration external to the cell causes water to pass through the cellular membrane into the external fluid, thus lowering the amount of internal water. This process causes the cell to shrink which of itself is not damaging to cell viability. It does, however, greatly lessen the chances of large intracellular ice crystals forming thereby rupturing the cell. This osmotic change in cell water requires a minimal amount of time. When cells are frozen so quickly that there is insufficient time for liquid water to leave the

cell before freezing, the percent of viable cells remaining is greatly reduced. This discovery leads to the realization that instantly submerging cellular material in liquid nitrogen, having a boiling point of -196° C., does not result in a high survival rate. If cells are frozen so quickly that they undergo little or no shrinkage due to water loss they will not survive.[10]

These discoveries have led to the development of the "two-step" cooling procedure in which cells are protected from freezing injury by what has been termed "prefreezing," that is, cells time-lap frozen to a subzero temperature, for example, -20° C. for a short period followed by a subsequent rapid freezing to storage temperature.[11,12] The extracellular freezing during the prefreezing stage provides sufficiently high salt concentrations to shrink the cells and avoid or reduce intracellular ice formation on subsequent cooling. The larger the cell the more slowly it must be cooled to avoid intracellular ice formation.[13] See Figs. 29 and 30.

CRYOPROTECTIVE AGENTS

Cryoprotective agents are chemical substances which inhibit the formation of ice crystals thereby affording some degree of protection against freezing. All cryoprotective agents have certain features in common among which is a high solubility in water. As freezing begins water is removed from solution (as ice) with a resulting increase in concentration of dissolved solids. A high solubility for an added substance assures that crystallization of that substance will not occur shortly after freezing begins.[10]

A second requirement for a cryoprotective agent is that it have low toxicity to the cells it is protecting. Some cryoprotective agents have been chosen not only because they satisfy these requirements but have as well a high molecular weight, not permitting

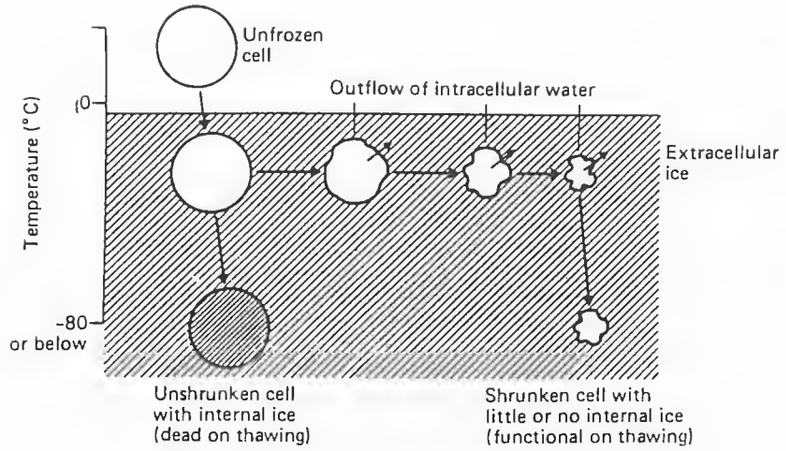

Fig. 29

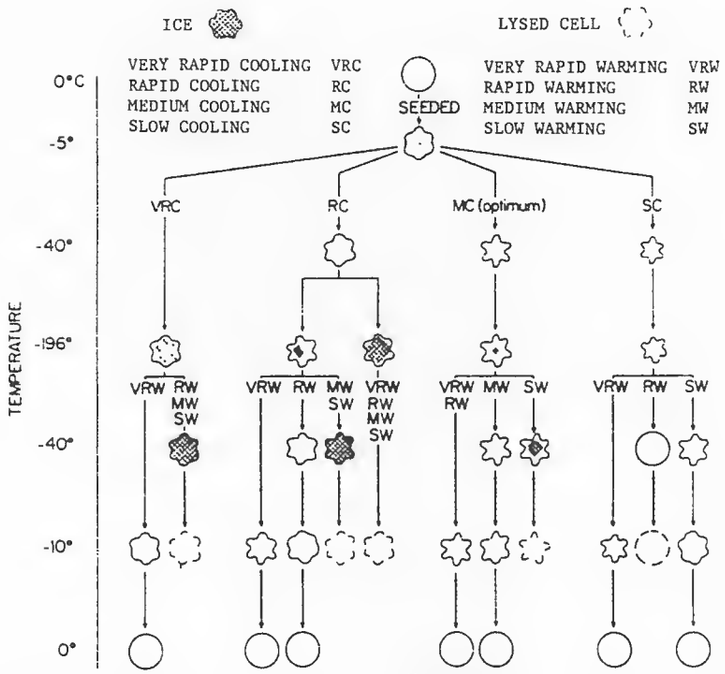

Fig. 30

them to enter cells. These substances are polymers, that is, long chains formed of a repeating unit linked together. An example of such a polymer is polyvinylpyrrolidone which has also been used as a blood plasma substitute.[14,15]

The greatest protection is afforded by cryoprotective agents to cells that are slowly cooled rather than to those rapidly cooled. As the concentration of the agent is increased more protection is given at lower rates of cooling. It is believed that cryoprotective agents reduce the proportion of the system that is converted into ice.[16] Less extracellular ice prevents an excessive increase in the concentration of dissolved solids (electrolytes).[16]

Another way of interpreting the protective action of these agents is that as ice is formed the concentration of the cryoprotective agent increases rather than the more toxic electrolytes (salts). The use of the protective agent moderates the rise in salt concentration so that sufficient cell shrinkage will occur to minimize problems with intracellular ice during rewarming, an aspect of cell preservation discussed in greater detail below.

Two frequently used cryoprotective agents are dimethylsulfoxide (DMSO)[17] and glycerol (glycerine).[18] The use of a permeating agent, for example, DMSO requires a lower pre-freezing temperature for optimal protection as the concentration is increased. Toxic injury to cells by solutes (including dissolved salts) at high concentrations is reduced at low temperatures. Addition of the cryoprotective agent to the cell suspension at 0^{o} C. minimizes direct chemical toxicity.[10]

THAWING OF CELLS

The manner in which cells are thawed is equally as important as the manner in which they are frozen. In general, it has been

found that rewarming as rapidly as possible leads to optimal survival.[14] The finding that rapid thawing can protect cells against the injury associated with rapid cooling indicates that the injury associated with intracellular ice occurs at least partially during rewarming and not during the initial formation of ice on cooling.[12,19]

The damage done to cells during rewarming is caused by the recrystallization of ice onto crystals of ice (intracellular) already present. As some water forms it remains below 0^{o} C. and can increase the size of existing ice crystals thereby causing the same type of damage which may be found during freezing. For these reasons rapid warming (by agitating the sample in a 37^{o} water bath) is safer and results in less cell death than slow warming.[14] If a cell contains too much intracellular ice no increase in thawing rate can avert its destruction.

When cells are cooled slowly they shrink by losing water. If these slowly frozen cells are then warmed slowly survival rates are high because they rehydrate slowly as liquid water gradually returns to the cell during melting. It is seen, then, that the rate at which cells are thawed is, for optimum survival, determined by the rate at which they were frozen.[13]

POST-THAW PROCESSING OF CELLS

The most important post-thaw stress has been that of the removal of a permeating cryoprotective additive from the system. This is usually carried out by dilution, either continuous (dialysis) or abruptly. Some cell types will tolerate abrupt dilution better than others and the presence of serum in the diluting media may reduce the trauma.[20]

The temperature at which the dilution is made is significant, an abrupt dilution being tolerated better at room temperature (or at 37° C.) than at 0°. Thawing is a dilution process.[20]

CELL CULTURE

The culturing of cells is a technique whereby individual cells are made to replicate under artificial conditions (in vitro) while being supplied with all necessary nutrients. The container is usually a sterile Petri dish placed in an incubator held to some optimum temperature. The cells are submerged in a nutrient medium providing such required substances as amino acids, nucleotides, sugars, vitamins and other necessary cofactors. Commonly, an appropriate antibiotic is also added to prevent the unwanted growth of other organisms. A contaminating organism expecially difficult to eliminate are the mycoplasmas. Years of painstaking work may be required to develop a nutrient medium which will support the proliferation of a given cell type. For example, insect cells could not for many years be cultured until an appropriate medium was developed.

For some organisms a controlled atmosphere is required having a specific oxygen-carbon dioxide ratio. Special apparatus has been developed capable of providing any particular atmosphere desired, with or without agitation.

Cells are quite often grown in the presence of radio-tagged factors (isotopically labeled) which are incorporated into certain biosynthesized substances, for example, DNA, RNA, protein or carbohydrates. The radioactivity they carry makes possible the detection of the substances the labeled material has entered.

All normal cells grown under culture conditions (except cancerous cells) follow a similar pattern of growth. Following a

short period of several hours (lag phase) a new culture will grow at a logarithmic rate, this stage being known as the "log phase." After a period of such rapid growth the nutrients provided begin to diminish and toxic byproducts accumulate (alcohol, for example, in some cells). The growth rate diminishes and some cells die either from the presence of toxic substances or lack of nutrients. This stage, in which the number of live cells remains essentially constant, is known as the "stationary phase."

With the decline of nutrients further and the accumulation of more toxic metabolic products the cells enter the "death phase" which culminates in the death of all the cells.

However, this process of cell culture may be extended indefinitely simply by removing a small number of cells and placing them in a new nutrient medium, providing the proper conditions for optimum growth. Thus, through cell culture there is created the illusion of immortality which was dramatically demonstrated by the French biologist, Alexis Carrel. In an experiment which lasted 32 years Carrel kept alive under continuous culture the cells from an embryonic chick heart.[21] This experiment could have been continued indefinitely if desired.

Another factor operates in cells under culture which is not related to either the exhaustion of nutrients or the accumulation of toxic byproducts. As mammalian cells divide they remain in contact with each other and eventually become overcrowded in a limited space. Even though the nutrients are replenished continued cell growth (of a given population) cannot be maintained and will eventually cease. This phenomena was described in greater detail previously (Chapter II) and results from contact of specific cell recognition receptors on the cell surface with adjacent cells. The contact of specific lectins with matching glycoproteins generates cytoplasmic signals which terminate the replicative process within

the cell. When a new culture is begun from an old one, cell-to-cell contact is broken and cell division begins once again.

With cancerous cells division does not cease simply from cell contact or overcrowding within the culture. As long as nutrients are provided cancerous cells will continue to multiply. Because they continue to proliferate uncontrollably it appears that there is a defect in the cell recognition mechanism which would otherwise terminate cell division. The presence of this defect (cell recognition and adhesion) also explains why cancerous cells metastasize, that is, lose contact with their neighbors and migrate to other parts of the body thereby growing into additional cancers.

What has been described above relating to cell culture has great implications for live cell therapy. In the present practice of cell therapy tissues are taken from a fetal animal, dissociated and preserved for future use. With the introduction of cell culture techniques dissociated fetal cells would be placed under culture conditions and maintained indefinitely until needed.

It was pointed out above that the development on the cell surface of specific recognition receptors follows a timed program which in detail, is largely unknown. By employing cell culture the optimum age of each fetal cell type could be determined quite easily, something which would be impractical through the use of fetal tissues directly.

In cell cultures of fetal rat liver (hepatocytes) alpha-fetoprotein was produced during the log phase (most proliferative stage) but disappeared during the stationary phase. The beginning of a new culture marked the reappearance of alpha-fetoprotein synthesis.[22] Following live cell injection the cells are essentially under culture conditions (37° C.) and provided with a nutrient medium. Until this dissolution as cells they are capable of synthesizing alpha-fetoprotein which acts as an inhibitor of the immune

response to foreign substances. Freeze-dried cells may not be capable of carrying out the biosynthesis of this important protein, depending on the method of cryopreservation used (if employed at all). Freeze-dried cells (unless properly frozen and thawed) have been ruptured, releasing highly antigenic material thereby stimulating the immune system. It is for these reasons that practitioners of cell therapy using freeze-dried cells are obligated to supplement the injections with antiinflammatory drugs (for example, cortisone or cortisol) to prevent a massive immune response jeopardizing the outcome of the therapy.

In the future live cell therapy may be practiced, not by dissecting a fetal animal, but by withdrawing from a permanently maintained cell culture the desired cell type, in the desired amount and of the proper age for therapeutic use.

Ongoing research at the American Biologics hospital in Mexico is investigating the many aspects described above related to improving the practice of live cell therapy.

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